

DIETARY FIBRE METABOLISM  
AND  
COLON FUNCTION

by

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A thesis submitted for the Degree of Doctor of Philosophy,  
University of Edinburgh, August, 1979.



## ABSTRACT

Dietary fibre is a complex material made up of plant cell polymers which are not digested by gastrointestinal enzymes of man but partially fermented by the commensal bacteria in the colon. Since in man most of the colon is not readily accessible, indirect methods must be sought to study dietary fibre metabolism and its effects on colon function. In this project gas chromatographic measurements of two of the end products of fermentation,  $H_2$  and  $CH_4$ , which are expired in the breath were used to monitor fibre degradation in the colon. Effects of fibre on colon function were assessed by measuring changes in stool weight, faecal constituents, transit time and serum lipids. The effect of dietary fibre ingestion on bile acid metabolism and the role of the colon in this process was further studied in the rat.

Preliminary studies on the normal excretion pattern of the two gases showed that breath  $H_2$  was excreted at a level of less than 1.0  $\mu\text{mol/l}$  and follows a regular daily pattern of excretion. The concentration was decreased by fasting.  $CH_4$  excretion was limited to certain individuals, about a third of the subjects studied, though the proportion of excretors increased with age. In those who excreted  $CH_4$  there was no regular daily variation in the excretion and levels were unaffected by diet. Test meals of the dietary fibre components, in the manner administered here, reached the caecum within three hours.

Short term separate administration of chemical isolates of fibre resulted in raffinose, stachyose and hemicellulose increasing total breath  $H_2$  excretion from a base line level of  $1.73 \pm 0.73 \mu\text{mol/l}$  to levels of up to  $16.36 \mu\text{mol/l}$ . Cellulose, pectin and lignin did not alter the excretion level.  $CH_4$  excretion was unaffected by any of the polymers. Difference in physical properties of the same chemical

polymer appear to have no influence on the  $H_2$  or  $CH_4$  production.

The ingestion of 200g raw carrot daily for three weeks increased  $H_2$  evolution significantly but had no effect on the  $CH_4$ . There was a lag period of a few days between the start of carrot intake and the increase in breath  $H_2$  excretion. Carrot decreased the serum cholesterol concentration from a control level of  $6.6 \pm 0.5$  to  $5.9 \pm 0.3 \text{ mmol/l}$  ( $P < 0.05$ ) and more than doubled bile acid excretion, particularly primary bile acids. The effect on cholesterol and bile acids remained the same three weeks after cessation of carrot intake. The effect on stool weight was modest and made no impact on the transit time.

The addition of cereal fibre to the diet of rats affected the metabolism of bile acids in the gastrointestinal tract both quantitatively and qualitatively and their synthesis in the liver.

It is concluded that dietary fibre because of its physical properties of absorption, adsorption and cation exchange and the partial degradation of some of its component polymers by bacteria in the colon, alters the distribution of organic and inorganic substances in the gut which change the luminal content of the colon. This change in luminal content affects the activity of the bacteria and influence some physiological functions of the colon like motility, water and electrolyte absorption and secretion, bile acid metabolism and reabsorption and cholesterol degradation and excretion. Different polymers of dietary fibre have different effects on each of these variables. It is possible to examine indirectly fibre metabolism and its effects on colon function by measuring expired gases and stool constituents.

#### DECLARATION

This thesis has been composed by myself and the work included has been done by me, except for the things duly acknowledged in the text and the rat bile acid method, for the development of which the help and expertise of Mr. Gordon Brydon, Senior Biochemist, was invaluable.



### ACKNOWLEDGEMENTS

I am indebted to the World Health Organisation for giving me the opportunity to study in this country and the extra financial support to finish this project.

I am very grateful to Dr. M. Eastwood for giving me access to the facilities in his laboratory, for his guidance and continued interest in my project, and for his understanding and patience. To Dr. R. Passmore, for his direction, the many constructive criticisms, the support and for those encouraging words which brightened gloomy moments.

My appreciation and thanks goes to all past and present members of the Wolfson Gastrointestinal Laboratories, particularly Mr. G. Brydon, Mrs D. Smith, Dr. J. Robertson, Mr. A. Smith and Mrs F. Wemyss, for their unreserved co-operation, unlimited technical aid and most of all their friendship.

I would also like to thank the Department of Biochemistry for keeping the rats and helping with the analysis of the liver cholesterol 7 $\alpha$ -hydroxylase enzyme; the Department of Medical Illustrations and Photography (W.G.H.) for their invaluable aid in producing the illustrations; Mrs S. Helliwell of the Metabolic Ward, for taking the dietary histories; Mrs L. Dodds for her unlimited co-operation and patience while typing this thesis; and finally all my friends for their continued moral support.

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ABBREVIATIONS USED

AACC	-	American Association of Cereal Chemists
BTPS	-	Body Temperature and Pressure (Saturated)
C	-	Cholic Acid
CDC	-	Chenodeoxycholic Acid
DC	-	Deoxycholic Acid
DF	-	Dietary Fibre
FAK	-	Flako Autumn King
FEV <sub>1</sub>	-	One second Timed Vital Capacity
FID	-	Flame Ionization Detector
GLC	-	Gas Liquid Chromatography
GSC	-	Gas Solid Chromatography
HDC	-	Hyodeoxycholic Acid
KD	-	Katharometer Detector (Thermal Conductivity Detector)
LC	-	Lithocholic Acid
MC	-	Muricholic Acid
MCTT	-	Mouth-to-Caecum Transit Time
NDC	-	Nor-deoxycholic Acid
SITT	-	Small Intestinal Transit Time
SST	-	Single Stool Transit Time
TLC	-	Thin Layer Chromatography
TMS	-	Trimethylsilyl
UDC	-	Ursodeoxycholic Acid
$\dot{V}_E$	-	Respiratory Minute Volume
V <sub>T</sub>	-	Tidal Volume
VFA	-	Volatile Fatty Acids

CHAPTER I

I N T R O D U C T I O N

## INTRODUCTION

### Towards a Definition

It is appropriate in the first instance to define dietary fibre. This, of course, is difficult because of the lack of a general consensus amongst workers in the field. However, an account of the attempts by different workers towards a definition may help to clarify some of the points that will be raised in this thesis. Changes in the definition became necessary with more knowledge of the chemical nature of the substances which constitute fibre and of their disposal in the alimentary tract.

The complex substances which we now identify as dietary fibre and their effects on the gastrointestinal system have been appreciated for centuries (McCance and Widdowson, 1956). Hippocrates (430 B.C.) is reputed to have said "whole meal bread clears out the gut and passes the excrement, white bread is more nutritious; it makes less faeces". Galen (c. 160), graded the then present four kinds of wheat flour according to their nutritive and faecal bulking value; the more refined the wheat flour, the more nutritious it is and less faeces formed. Though the word fibre was used since 1682 (Trowell, 1977) it was only in the early nineteenth century that proper chemical analysis and chemically defined names were assigned to identify the entity fibre. Horsford (1846, 1854), first in the famous Liebig Laboratory and later at Harvard, did the preliminary analysis on fibre. The term "crude fibre" was then introduced by Henneberg and Stohmann (1860) to describe the substance remaining as residue when plants are treated with 1.25% sulphuric acid, 1.25% potash, water, alcohol and ether (Mangold, 1934). Despite the crudity of the method of analysis employed, to provide the basis for the definition, this term was wide accepted by



nutritional scientists particularly those in animal nutrition, where it is still popular and enjoys legal backing. As the significance of fibre in human nutrition and gastrointestinal function became better appreciated, suitable definitions for medical and human nutritional uses were required. One of the first contributions towards this demand was made by McCance and Lawrence (1929). They named the substance as 'unavailable carbohydrate' and the term was widely used in medical circles until about 1970. Though most of the chemical polymers of fibre are carbohydrates, they are not the only chemical constituents. Also, though the carbohydrates in fibre may not be available in the usual nutritional sense, i.e. not digested by endogenous enzymes in the upper parts of the gastrointestinal tract nor absorbed from the small intestine, it is not altogether unavailable as it is metabolised by bacteria in the colon and some of the bacterial byproducts are absorbed and utilised. Other terms, besides 'unavailable carbohydrate', used by lay and medical persons alike, are 'roughage' and 'bulk'. These names are misleading and unsatisfactory for present day use.

A more precise and suitable definition became necessary with the increasing understanding and knowledge about the chemical composition, metabolism and the role of these complex polymers. Hipsley (1953) had suggested the term 'dietary fibre' in place of 'unavailable carbohydrate', but this new term failed to get wide acceptance for many years. Trowell (1972, 1974) revived the term and defined it as "the skeletal remains of plant cells that are resistant to digestion by enzymes of man". This definition was later modified to include undigested storage polysaccharides with associated lipid and nitrogen-containing substances and other yet unidentified substances of plant cells (Trowell et al, 1976). The

term 'dietary fibre' as defined by Trowell, is now widely accepted and in common use, yet it does not satisfy all requirements. The word 'fibre' is not a true reflection of the structure of the polymers and not all sources of fibre are dietary. Spiller (1977) had suggested the term 'plantix' with definition essentially the same as that forwarded by Trowell. Again, 'plantix' has its own disadvantages, because not all dietary fibres are of plant origin as implied by this term. Synthetic and pharmaceutical preparations are neither dietary nor entirely of plant origin, but may function as dietary fibre. There are also suggestions that some components of animal connective tissues, which are not digested by human intestinal enzymes, should be considered as part of the broad concept of dietary fibre. Godding (1976) had proposed the term 'edible fibre' instead of 'dietary fibre' or 'plantix' to cover all the described varieties. Though this term still carries one of the weaknesses of dietary fibre, i.e. the word 'fibre', some workers seem to prefer adopting it (Trowell et al, 1978).

Even if agreement on a comprehensive term is difficult at the moment, it should now be possible to provide a chemical description of the substances. But, as the preceding outline indicates, the components of these mixtures of complex polymers depends to some extent on the definition accepted. However, there is at least general consensus as to the major components and this makes chemical description feasible.

What then constitutes 'dietary fibre'. The main chemical components are given in the table below:-

Table Ia. Components of Dietary Fibre

(based on Southgate, 1978)

<u>Major Groups</u>	<u>Principal Structural Types</u>	<u>Amount</u> (%)
A. <u>Structural</u>		
Cellulose	B-D Glucan	15-40
Hemicelluloses	Xylans (arabino, glucurono) Mannans (gluco, galacto) Galactans (arabino)	15-30
Pectins	Galacturonans	1-4
Lignins	Aromatic polymer	6-25*
B. <u>Non-structural</u>		
Pectins	Galacturonans	10-30
Oligosaccharides	Raffinose (glu, fru, gal ) Stachyose (glu, fru, gal , gal )	
Gums	Glucuronic, galacturonic acid with neutral sugars (xylose, arabinose, mannose)	
Mucilages	Gluco and galactomannans	

\* This value is rather high and may be due to artefacts as a result of the relatively inaccurate method of lignin estimation.

Other fibre constituents which are present in different plants in varying amounts are waxes, cutins, storage polysaccharides like inulin and associated substances like phytate, protein, silica, chromium, zinc, etc. The other important group of polymeric fibre components found as major constituents of seaweeds, used as foods by some communities and in small amounts in the food and pharmaceutical industries, are alginic acid, agar and carrageenans (Cummings, 1976; Southgate, 1976).

This is current state of fibre definition. In the following text the term 'dietary fibre', despite its drawbacks, is used and the definition given by Cummings (1976) is followed. This is "dietary fibre is a group of substances of plant origin, which are found largely but not entirely in the plant cell-wall and which are thought to be neither digested nor absorbed in the upper gastrointestinal tract".

## METABOLISM OF DIETARY FIBRE

### Historical

Before the beginning of the nineteenth century, it was generally assumed that dietary fibre was not digested at all and passes through the gastrointestinal tract unchanged. As reported by Mangold (1934) in his authoritative review on the digestion and utilisation of crude fibre, it was Sprengel (1832) who first observed fibre digestion in ruminants. This observation was later subjected to exact chemical study by Henneberg and Stohmann (1860). They fed cattle with different high fibre diets and by measuring input and output determined the digestibility of the different dietary sources and the factors which influenced the digestibility. The results showed that meadow hay feed was digested to the extent of 57-65% and oat straw 55%. Digestibility varied between individual cattle and was influenced by the other constituents of the feed, like protein. From this study the investigators were able to suspect the occurrence of soluble intermediary products during digestion of the fibre. Ellenberg et al (1915) showed the bacterial nature of the rumen digestion by demonstrating that a mucosal extract of the rumen was unable to degrade dietary fibre. Hofmeister (1880) found that dietary fibre was also degraded in the caecum and colon. Again, the nature of this caecal and colonic degradation was demonstrated to be bacterial by Scheunert (1906), who compared incubation of fibre with mucosal extracts from the colon and caecum, and bacteria from the respective areas. The extracts were unable to degrade fibre while the bacteria were able to.

In the pig, it was shown that digestion of dietary fibre occurred in the caecum and colon, but not in the stomach or small intestine (Krzywanek, 1929). This finding lead to the suspicion

that dietary fibre was digested in animals other than ruminants. Many experiments on different omnivorous and carnivorous species have now established the expectation. By using similar experiments to the one described above, the degradation has been shown to be bacterial.

In man, the first recorded worker to have studied digestion of dietary fibre appears to be Weiske (1870). He studied the digestibility of carrot, celery and cabbage in two subjects and found that the digestibility coefficient of these dietary fibre sources for the two volunteers were 47.3 and 62.7% respectively (Mangold, 1934). Gosman and van de Reis (1925), extending this observation, showed that cellulose was broken down by bacteria from the faeces, colon and small intestine of man. Though there were other studies on dietary fibre digestion in man at the beginning of this century, the first systematic investigation was by Williams and Olmsted (1936). These investigators studied three male medical students in a metabolic ward for many weeks. Known amounts of dietary fibre extracted from ten common dietary sources and a control fibre-free diet were administered. The percentage disappearance of each component, the products of bacterial digestion, factors that influenced digestibility of the fibre and the effect of fibre source on stool weight were measured. The findings in summary were:-

- (1) Hemicellulose was the most metabolised, cellulose intermediate and lignin the least.
- (2) The amount of lignin present in the dietary fibre source affected the digestibility of the other components.
- (3) The percentage disappearance of any one component was similar for the three subjects.
- (4) The more dietary fibre source that was digested the greater the stool weight.
- (5) The quantity of volatile fatty acids (VFA) in the stool was directly proportional to the amount of dietary fibre digested.

These findings, with very few discrepancies, have been confirmed by several workers (Sealock et al, 1941; Hunnel et al, 1943; Southgate and Durnin, 1970; Southgate et al, 1976b).

Between 1940 and 1960 studies on dietary fibre in man were first concerned with cereal fibre (bran) and its effects as a laxative (Fantus et al, 1941; McCance and Widdowson, 1942; McCance et al, 1953). Later interests shifted to the effect of dietary fibre, particularly vegetable fibre and pectin, on serum cholesterol (Keys et al, 1960; Grande et al, 1965; Eastwood, 1969). This led to a greater interest in research on dietary fibre and the trend continues. More precise definitions, better methods of analysis and standardisation of research have been attempted. The investigation of Southgate and Durnin (1970) is of particular interest and significance in this respect. They studied 49 Glaswegians of both sexes with ages ranging from 15-85 years. The volunteers ate two controlled diets in turn; one with a high and one with a low content of dietary fibre. Intake and faecal excretion of two main dietary fibre components (pentosans and cellulose) were measured over a period. The results are in general agreement with those of Williams and Olmsted's except for the individual values. Contrary to the findings of Williams and Olmsted, Southgate and Durnin found wide variation in the gastrointestinal disappearance of the dietary fibre components in different individuals. This may be explained by the number of subjects in the two studies - 3 males in the former and 49 of varying age and sex in the latter. Another study by Southgate et al (1976a) on bran in a metabolic ward has also confirmed most of the earlier findings.

Of the individual components of dietary fibre, cellulose has been

most studied. There is a wide range of individual variation in cellulose digestion, of the order of 0-80% (William and Olmsted, 1936; Southgate and Durnin, 1970; Milton-Thompson and Lewis, 1971). An average of 40% is an accepted value. Hemicellulose is digested to a greater extent than cellulose and with it too there is a wide range of individual variation. Williams and Olmsted (1936) found a digestion rate of  $56 \pm 2\%$  and Southgate and Durnin (1970)  $87.2 \pm 9.2\%$ . The fate of pectin in man is not clear. In one study about 95% digestion was reported (Werch and Ivy, 1941). Though Williams and Olmsted in their study showed some digestibility of lignin, it is generally agreed that lignin is completely undigested in the human gastrointestinal tract (Cummings, 1973) and Williams and Olmsted's findings may be due to their methods. The digestibility of the other components of dietary fibre is largely uninvestigated.

#### Factors Affecting Dietary Fibre Digestion (Fermentation)

Since dietary fibre is a complex material made up of different chemical polymers, the relationship of these polymers within the complex structure and their interaction with the external environment, would be the two determinants of the level of degradation.

Each polymer has a different chemical structure which governs its chemical reactions and physical properties (Southgate, 1976). Cellulose is a linear monopolysaccharide insoluble in water but

susceptible to acidic and enzymatic hydrolysis.

Hemicellulose, on the other hand, is a complex mixture of branching polysaccharides variably soluble in water and dilute alkali, and relatively resistant to enzymatic hydrolysis (Southgate, 1976).

Pectin is a heteropolysaccharide mainly composed of galacturonic



acid, soluble in hot water and easily hydrolysed by enzymes and acid; while lignin is an aromatic polymer insoluble in water and highly resistant to chemical hydrolysis (Cummings, 1976). Thus, the relative quantity of each polymer and the presence or absence of one component or another alters the behaviour of the complex and its degradation. For instance, a dietary fibre source rich in lignin content would be expected to be less degraded compared to a source with high hemicellulose or pectin content. Williams and Olmsted (1936) have demonstrated that dietary fibre sources with high lignin content (cotton seed hull, wheat bran, alfalfa leaf) were digested less when compared with those sources with low lignin content (carrots, corn germ, peas, beet pulp, cabbage). These investigators and also Cymbaluk et al (1973) have found that the degradation of cellulose is affected by the degree of lignification of the plant. It appears therefore that the presence of a high lignin content affects not only the overall digestibility of the dietary fibre complex but also the degradation of each individual polymer. The presence of silica and cutin have also been found to decrease the digestibility of the other dietary fibre components (van Soest and Jones, 1968; Keys et al, 1970). Furthermore, some variation in the chemical structure of one of the polymers may alter some of its usual properties so as to reflect on the behaviour of the fibres as a whole or on the other individual polymers. Though in vivo studies in this line are lacking there are suggestive evidences from in vitro studies. For example, properties of pectin like solubility, gelling, etc. are dependent on the degree of methylation of the carboxylic acid group; highly methylated pectin is an excellent gel former, but not poorly methylated pectin. Also a highly substituted hemicellulose is less soluble than one with few



substitutions (Southgate, 1976).

In the gastrointestinal tract, there is interaction between the fibre polymers and the surrounding solutes, other dietary substances, various metabolites, and the intestinal bacteria (Eastwood, 1975). Each of these variables would presumably modify the metabolism of the fibre. To cite a few examples, the pH of the surrounding fluid could affect the different properties of the constituent polymers and in this way modify the dietary fibre metabolism (Bown et al, 1974). Gel formation may make the core of the dietary fibre inaccessible to bacterial enzymes (Southgate, 1978). Salt formation of some of the polymers may make them resistant to hydrolysis. A high concentration of bile acids in the colon, as a result of binding in the small intestine, may be harmful to the bacteria, and so decrease their activity on the dietary fibre (Floch et al, 1971). Specific bacteria have specific enzymes for metabolising different components of dietary fibre (Hungate, 1976; Prins, 1977) and individuals to some extent differ in their gastrointestinal bacteria (Gall, 1968; Holdman et al, 1976). Thus, the presence or absence of a specific bacteria in the individual concerned may determine the degree of breakdown of a certain dietary fibre source.

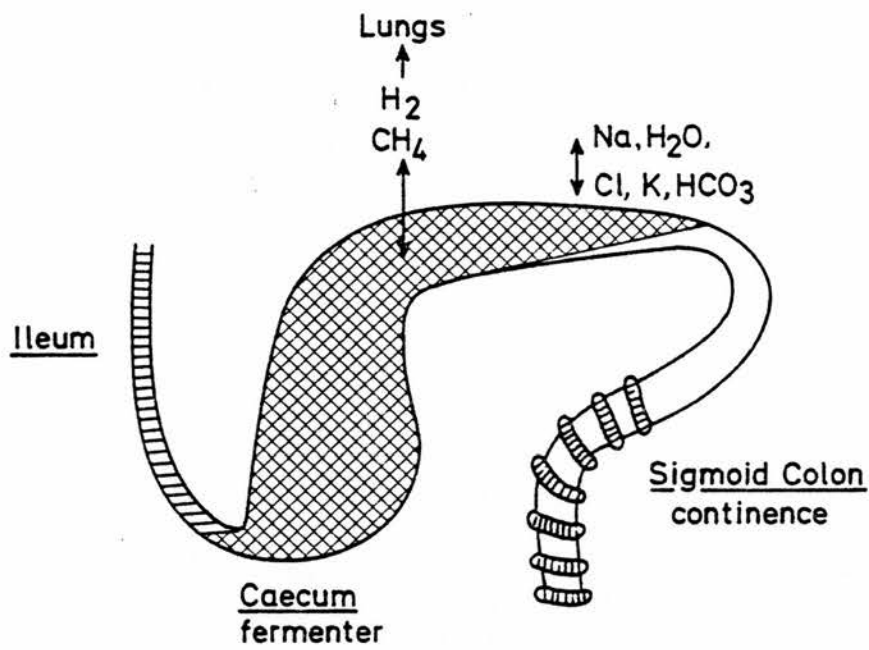
In short, the source of the dietary fibre, the relative quantity and the chemical nature of each polymer, the interaction of the polymers with surrounding solutes and organic materials, the nature and status of the bacteria, determine the level and extent of dietary fibre degradation in the colon.

#### Colon Function

The large intestine in adults is approximately 1.2m long and includes the caecum, ascending, transverse, descending and sigmoid

colon, the rectum and the anal canal. In terms of degree of function the large intestine can be divided into right and left sides. The right side of the colon and the caecum are the site for most of the fermentative, absorptive and secretory activities, while the left side is mainly for the storage of the residues of food and excretion products, which are disposed of at intervals (Fig. 1a). The luminal content of the colon consists of bacteria, undigested food residue with variable amounts of water and electrolytes, shed mucosal cells and different secreted and excreted substances. Colonic activity and function is governed by the interactions of these various components. The bacteria, particularly, play a central role because of their diversity and the wide range of metabolic activities that they perform. Some of their metabolic activities which have bearing on colon function are considered below.

The number of bacteria in the colon is very large. In the sigmoid colon and rectum there are about  $10^{11}$  bacteria/g of wet weight, while in the small intestine the number is less than  $10^4$ /g. Ten to twenty percent of the faecal dry mass is constituted of bacteria (Gall, 1970; Drasar and Hill, 1974). In the normal adult colon the resident bacterial flora consists 96-99% anaerobes and about 45% of these are reported to be Bacteroides fragilis (Moore et al, 1969; Hill and Drasar, 1975). The other major species found are Bifidobacterium adolescentis, Eubacterium aerofaciens, Escherichia coli, and to a lesser degree Streptococcus viridans, Streptococcus salivarius, and several species of lactobacilli and clostridia (Hill and Drasar, 1975). There is a wide range of quantitative difference in the bacterial flora between individuals in the same population group and some qualitative difference between different population groups. Some African and

Figure - IaTHE FUNCTIONS OF THE COLON

Asian populations have been found to have less bacteroides and no NDN +ve clostridia as compared to some European population (Gorbach et al, 1967; Aries et al, 1969; Borriello et al, 1978). Though population groups with big differences in dietary habits and way of life show differences in their bacterial flora, short term dietary manipulation in an individual or in a population group is not accompanied by significant quantitative and gross qualitative changes in the intestinal flora (Drasar and Jenkins, 1976). All the above cited observations are from studies on faeces or contents from easily accessible parts of the colon. Some differences in the bacterial flora would then be expected in the different parts of the colon. One of the very few studies which attempted to answer this question is one by Bentley et al (1972). They collected intestinal contents from the ileum, caecum and transverse colon separately, by using needle aspiration during abdominal operations. They investigated the bacterial flora both quantitatively and qualitatively and found that the flora in all the regions tested were similar to that of the faeces though the number varied, increasing caudally. A study of bacteria in different regions of the colon soon after death demonstrate the same (Moore et al, 1978).

Amongst the major metabolic activities performed by colonic bacteria are anaerobic fermentation of carbohydrates, proteins, fats and intestinal mucin; degradation of bile acids, cholesterol, neutral steroids, phospholipids and bile pigments (Drasar and Hill, 1974; Hungate, 1976). With respect to carbohydrate fermentation, the bacteria in the colon are not only able to metabolise those carbohydrates usually digested by man, e.g. monosaccharides, disaccharides and starch, but also have enzymes for breaking down a number of oligosaccharides, polysaccharides and glycosides for

which man and other mammals have no enzyme. Amongst these are raffinose, stachyose, pectins, hemicelluloses, cellulose, polygalactose, polymanose, etc. (Drasar and Hill, 1974). The fermentation products of dietary fibre carbohydrates in the colon are similar to those in other situations and in vitro. They include the volatile fatty acids (VFA), mainly acetic (about 70%), propionic and butyric acids, and the gases, carbon dioxide ( $\text{CO}_2$ ), hydrogen ( $\text{H}_2$ ), methane ( $\text{CH}_4$ ) and others. (Mangold, 1934; Hungate, 1976). These partially degraded organic compounds are formed because anaerobic bacteria cannot metabolise nutrients beyond the pyruvate stage due to the absence of oxygen. Some of these substances act also as  $\text{H}^+$  ion acceptors, as does the formation of  $\text{H}_2$  (Prines, 1977). The compounds are released to the surrounding medium and overall this form of metabolism is inefficient in terms of energy production for the bacteria but can be useful for the host animal. In ruminants, VFA formed and released by the bacteria are a major source of energy, though  $\text{CH}_4$  production and eructation is a major source of energy waste and has been a fire hazard (Hungate, 1976; Czerkowski and Breckenridge, 1975; Stevens, 1978). Non-ruminant animals including man produced VFA in their colons, and are able to absorb and utilise them (Elsden et al, 1946; Dawson et al, 1964; Argenzio and Southworth, 1975; Cummings, 1975; McBee, 1977; Leng, 1978; Stevens, 1978). One investigation on wild porcupines with their natural diet showed that on the average about 16% (5-33%) of the maintenance energy was contributed by VFA absorption from colonic fermentation (Johnson and McBee, 1967). In the same study it was shown that about 95% of the VFA produced was absorbed into the portal blood system with 88% of this coming from the caecum alone. In the laboratory rabbit 10-12%

of the basal energy requirement could come from VFA produced in the caecum (Hoover and Heitmann, 1972) and according to Parker (1976) it may be even 30-40%. In the rat more than 5% of the daily energy requirement comes from caecal VFA absorption (Yang et al, 1970). In man, though there is evidence for production and possible absorption of VFA from the colon, its contribution towards the energy requirement is not clear (Williams and Olmsted, 1936; McNeil et al, 1978). It could only be speculated that the contribution in individuals with comparatively low dietary fibre intake would not be significant, while in individuals with high intake it may be important. McNeil et al (1978) estimated that about 100kJ of energy could be derived from VFA produced by colonic fermentation of 20g dietary fibre consumed. This is about 1% of the basal metabolic rate and the implication in populations with scarce energy rich food intake but with high dietary fibre consumption (>100g/day) is obvious.

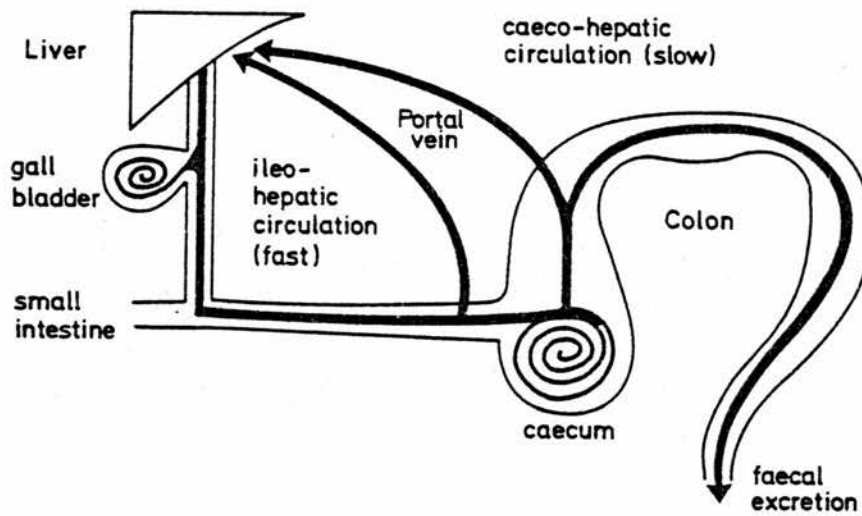
Colonic bacteria also metabolise bile acids, and this may have a bearing on colon function. Conjugated primary bile acids excreted in bile and reaching the small intestine are mostly absorbed unchanged into the enterohepatic circulation (about 90%) (Hardison et al, 1976; Sklan et al, 1976). The remaining 10% are deconjugated, dehydrogenated

and 7 $\alpha$ -dehydroxylated by intestinal bacteria mainly in the caecum (Heaton, 1972; Lewis and Gorbach, 1972; Hofmann, 1977). Some of the so-called secondary bile acids are then absorbed from the colon into the enterohepatic circulation and the remainder excreted in the faeces (Fig. 1b). The level of secondary bile acids produced, the type and the amount absorbed or excreted in the faeces will have a feedback effect on bile acid synthesis in the liver (Danielsson and Johansson, 1974; Hofmann, 1976).

Diet, including dietary fibre intake, may influence the amount and

Figure - Ib

THE ENTEROHEPATIC CIRCULATION OF BILE ACIDS



type of bile acid excreted by the liver and the proportion reaching the colon (Bergstrom and Danielsson, 1969; Pomare and Heaton, 1973). In the colon the level of breakdown and excretion is mainly determined by the bacteria but dietary fibre may also play a significant role.

The other major and well documented function of the colon is that of water and electrolyte balance. About 1.7 ml water, 0.28 mmol  $\text{Na}^+$  and 0.39 mmol  $\text{Cl}^-$  are absorbed per minute by the entire colon, while 0.03 mmol  $\text{K}^+$  and 0.18 mmol  $\text{HCO}_3^-$  are secreted (Levitan, 1962). The capacity is much more than this and as much as five litres of water can be absorbed per day (Debonnie and Phillips, 1978). Most of these activities are performed on the right side (Levitan, 1962; Devroede and Phillips, 1969). Other secretory activities, e.g. hormone secretion, are recognised but not yet sufficiently understood to make a reasonable discussion possible (Moxey, 1978).

Thus, the colon in man is a relatively inaccessible part of the gastrointestinal tract, with a diverse bacterial flora. The bacteria ferment non-digested or non-absorbed nutrients as well as metabolise a wide range of organic materials that are secreted or excreted into the lumen and reach the colon. The colon is also one of the major organs of water and electrolyte conservation and acts as a storage for waste products of food. The fermentative, metabolic, absorptive and secretory activities (occurring mainly on the right side) may be to a degree influenced by dietary fibre ingestion, either directly or indirectly.

#### Effects of Ingested Fibre on Colon Function

The effects of dietary fibre on the physiological functions at of the colon could be looked at from two aspects. The first is



the physical effect of undigested material reaching the colon and the second the direct and indirect effects of bacterial metabolites of the fibre.

The physical effects of dietary fibre are related to its chemical and physical properties. Studies in vitro indicate that while passing through the gastrointestinal tract fibre probably imbibes water and increases faecal bulk (Mitchell and Eastwood, 1976). Also dietary fibre from different sources vary in their water-holding capacity. For instance, potato fibre has a capacity of 2g of water/g and carrot fibre one of 23.4g water/g (McConnell et al, 1974). Cellulose takes up water well but lignin is a bad absorber. If what occurs in vitro also happens in the gastrointestinal tract, the increase in bulk would distend the wall of the colon and thus increase motility, thereby decreasing the transit time of the residue. This is current theory and may well be the case though there is no direct evidence. It is also possible that this water bulking effect may modify the absorptive and secretory activities of the colon. Dietary fibre has been shown to adsorb electrolytes and organic materials, particularly bile acids (Eastwood and Mitchell, 1976). Different dietary fibres and chemical components have different ion adsorption and bile acid binding capacity. Pear fibre, for example, has a weak cation-exchange capacity of the order of 0.6mmol/g, whilst turnip is a strong cation-exchanger, 2.3mmol/g (Eastwood and Mitchell, 1976). Brussel sprout fibre is a strong adsorber of bile acids while carrot fibre is weak (Eastwood and Hamilton, 1966). As a corollary, pectin is a strong mono-functional cation-exchanger and a poor bile acid binder, while lignin is a strong bile acid binder and a weak polyfunctional cation-exchanger (Eastwood and Mitchell, 1976). Thus, the differing

cation exchanging and bile acid binding capacities of dietary fibres are probably related to differences in their chemical composition. Because of these varied physical properties, different dietary fibre sources would be expected to modify colon function variably by changing the constituent of the effluent reaching the colon and altering the milieu of the lumen. This is achieved by acting as a carrier for a variety of substances from the upper parts of the gastro-intestinal tract as described above. Particularly, the adsorption of bile acids would be expected to change the entero-hepatic circulation with consequences on bile acid synthesis in the liver (Hofmann, 1976). Also, in vitro studies with bile acids and colonic bacteria have shown that increasing bile acid concentration inhibits bacterial growth (Floch et al, 1971). This may be important when an excess of bile acids are shifted to the colon. The ion binding capacity should be emphasised too, because of its sequestering effect and the possible attendant depletion of iron, calcium, magnesium and zinc (Haghshenass et al, 1972; Reinhold et al, 1976; Ismail-Bergi et al, 1977).

The other physical property of a dietary fibre which may have a bearing on colon function is a consequence of its original structure in the cell wall. The cell wall is thought to be made up of cellulose fibrils running in parallel as bundles, held together by hydrogen bonds. Each bundle is then interconnected by hemi-cellulose, pectin and lignin, to give a regular rigid pattern (Albersheim, 1975). This pattern might act as a matrix for bacterial activity and possibly facilitate colonic absorption (Partridge, 1976). Alternatively, fibres may entrap substances in their mesh and make them inaccessible for enzymatic and bacterial action (Southgate, 1978). These physical effects would be modified

by changes in mesh structure due to bacteria degradation of some of the chemical components. Hemicellulose, for example, though a good water absorber, is metabolised quickly by colonic bacteria and this would affect its potential bulking effect.

The effects of dietary fibre fermentation on colon function are probably mediated through chemical and gaseous byproducts of bacterial metabolism. These effects could act either locally or after absorption, through systemic mechanisms. Thus an increase of substrates as a result of dietary fibre intake might temporarily enhance bacterial growth and so change the degree and range of their luminal activity (Fuchs et al, 1976). There may also be a selective increase of certain species of bacteria related to the chemical composition of the fibre (Smith, 1965; Hattori and Hattori, 1963). The production of VFA may stimulate colonic motility and increase faecal bulk, both of which could decrease transit time (Cummings, 1973). It may also influence electrolyte absorption and secretion (Leng, 1978; Stevens, 1978). The production of different gases due to fibre fermentation by bacteria, not only distends the colon and stimulates peristalsis, but also may modify its absorptive and secretory activities by adding another physical variable to the luminal environment.

The polymers of dietary fibre absorb and adsorb substances while passing through the stomach, jejunum and ileum. Subsequent bacterial degradation of the binding polymer in the colon may release these substances. This would have an effect on the function of the colon, not only by changing the luminal content but also by altering the metabolism of the substance concerned. For instance, the release of bile acids in the colon and change to secondary bile acids, e.g. lithocholic acid, by the anaerobic

bacteria, would disturb the cellular function of the colon unless, of course, they are adsorbed to the bacteria (Gustafsson and Norman, 1969). Bile acids also have been found to influence water and electrolyte absorption by the colon (Forth et al, 1966; Dobbins and Binder, 1976). Similarly, other toxic substances may be left in the colon for a longer period of time after the carrier component is metabolised and be detrimental to the function of the colon. But, alternatively, toxic substances could either be diluted or their excretion into the faeces facilitated by increased dietary fibre intake (Burkitt, 1978). Here too, the effects may depend on the type of dietary fibre and its specific chemical composition.

So, dietary fibre primarily due to its physical properties of absorption, adsorption and cation-exchange, but also as a result of its bacterial fermentation, changes the contents of the lumen of the colon. These changes may have a direct or indirect effect on colonic function.

#### Gaps in the Study of Dietary Fibre Metabolism and Colon Function

Though some of the easily measurable effects of dietary fibre ingestion on gastrointestinal function, e.g. changes in stool weight, transit time and bile acid sequestration, are well documented, the mechanisms responsible for these changes are not clear. It is thought that the changes are a result of water absorption and bile acid adsorption by the fibre (Eastwood, 1975; Cummings, 1978b). This may well be so, but in order to define precisely the physical and chemical changes that accompany dietary fibre ingestion and its effects, the role of each component polymer separately and when it acts in concert must be understood. This requires systematic long-term studies in humans on controlled diets but this in practice is laborious and there are few such studies. The short and long-term

effects of dietary fibre intake have to be distinguished and specified.

The nature and controls of the caecal metabolism of dietary fibre and its effects on colon function, though very important, have been little investigated. This is due to the relative inaccessibility of the human colon and the lack of satisfactory knowledge on the normal physiological functions of the colon and its controls. There is also a lack of information on the effects of dietary fibre on the bacterial flora in the caecum or even in the faeces. The few studies on this are too contradictory to permit meaningful conclusions (Drasar and Hill, 1974; Fuchs et al, 1976). That the effects of dietary fibres on chemical substances either secreted or excreted into the intestinal lumen (e.g. bile acids), may be important in homeostasis is appreciated but the extent and significance are far from clear.

There is also a need for techniques to study the right and left side of the colon separately. This is because of the tremendous difference in activity of the two sides. The right colon, particularly the caecum, play a very active role in bacterial fermentation, absorption and secretion, while the left colon is more or less passive and a temporary storage of waste (Fig. 1a). Though the left colon is relatively accessible for a limited distance, the right colon is difficult to reach through the left because of its fixed nature and the number of flexures on the way. Intubation through the mouth is possible, but is beset by problems. First the distance from mouth to caecum is such that passing a tube will not be an easy exercise both to the subject and the investigator. Secondly, once the tube reaches the caecum, its effective usefulness as a sampler and dispenser is limited by the diameter of its lumen and by the consistency of the caecal contents. The luminal phases

(i.e. solid, liquid and gas) cannot be sampled proportionally so as to give a reliable picture of the metabolic processes. Thus, information about the accessible parts of the left colon has only limited use. At best, observations are approximations to or a static reflection of what had occurred during a dynamic process on the right side.

Individuals with an ileostomy or colostomy have been used to study dietary fibre metabolism (Holloway et al, 1978), but the bacterial flora of the intestine in such situations is quite different from normal intestine. Also, the stoma is exposed to atmospheric air, making strict anaerobic metabolism difficult. Thus, results from such investigations can hardly be extrapolated to the normal situation.

There is, therefore, a lack of information on several aspects of the function, effects and metabolism of dietary fibre requiring attention and detailed studies. New information should not only help in defining the properties of dietary fibre in terms of the chemical components but also be useful in the better utilisation of each component for a specified preventive or therapeutic purpose and the avoidance of those with undesirable effects. Until these questions are answered, meaningful assessment as to the merits and demerits of differing fibre intake will not be possible.

#### Possible Methods of Study in Man

As indicated above, the study of dietary fibre metabolism in man is hampered by the inaccessibility of the site where it mainly occurs, and limited by the range of manipulations that can be carried out in man. As a result indirect methods or animal experiments, with all their accompanied drawbacks, are resorted to. One such method of investigation is a balance study in a



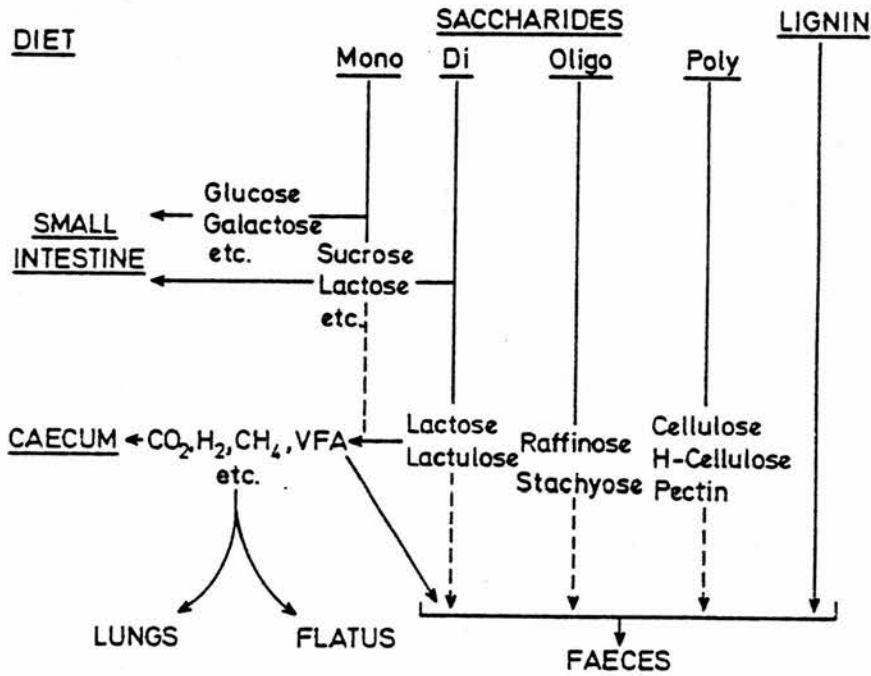
metabolic ward, where intake and outputs can be precisely measured. Such a study, besides being expensive both in time and money, is hindered by the long procedures needed for analyses of dietary fibre in foods and faeces (Van Soest and McQueen, 1973; Southgate, 1976). Also, balance study alone would only indicate the level of degradation of fibre and its effects on the metabolism of other nutrients and substances, but would not clarify the actual process.

Another way of studying any metabolic process is the study of its metabolic byproducts. This approach appears to be well suited for the study of dietary fibre, because some of the byproducts of its metabolism are readily accessible. As described earlier, the products of dietary fibre breakdown in the colon are VFA and a number of gases. These products could be measured in the faeces and flatus, or in the expired air after absorption from the gut and excretion by the lungs (Levey and Balchum, 1963; Calloway, 1968; Chen et al, 1970) (Fig. 1c). The gases  $H_2$  and  $CH_4$  are particularly suitable since they are not produced by mammalian cell metabolism and yet are two of the major products of anaerobic bacterial metabolism (Gall, 1968; Newman, 1974). Other gases, e.g.  $CO_2$  and  $N_2$  are not suitable since they are ubiquitous in the body and environment, unless specific identification methods are resorted to (e.g. radioactive labelling of precursors). Volatile fatty acids are absorbed into the inaccessible portal circulation and metabolised in the liver before reaching the systemic circulation and as such will not be available for measurement. Thus, the two gases,  $H_2$  and  $CH_4$ , are the best available indicators of the level of bacterial breakdown of dietary fibre in the colon.

The effect of dietary fibre on colon function could also be studied by measuring substances which are normally handled or

Figure - Ic

THE METABOLISM AND FATE OF DIETARY CARBOHYDRATES  
AND LIGNIN IN THE GASTROINTESTINAL TRACT



N.B.: H - Cellulose = Hemicellulose



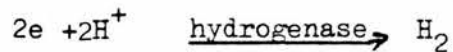
metabolised in the colon. These may include faecal water, electrolytes, bile acids, cholesterol, etc. Other useful indicators of colon function are faecal dry and wet weight, transit time and faecal bacterial floral changes.

All these variables may be employed separately or together to study dietary fibre metabolism and its effects on colon function indirectly. Different dietary fibre sources could be investigated as they occur naturally or individual chemical components be isolated and studied separately and in controlled combinations. In this project most of these possibilities are utilised but measurement of breath  $H_2$  and  $CH_4$  were depended on to assess dietary fibre fermentation in the caecum. The principle, development and application of this method is considered in greater detail below.

#### Breath Hydrogen and Methane Measurement

During metabolism of carbohydrates, fat and protein, organisms release free electrons and  $H^+$  ion, which need to be disposed of in one way or another, if normal homeostasis is to be maintained. In aerobic situations this is achieved with the help of a number of co-enzymes and finally the flavoprotein-cytochrome enzyme system by which the  $H^+$  ions are combined with  $O_2$  to form  $H_2O$ . Many strict and facultative anaerobic bacteria have neither the oxidant  $O_2$  available or the enzyme system to aid in the transfer (Gray and Gest, 1965). Thus, anaerobic bacterial metabolism of nutrients besides being less efficient in terms of energy output, predisposes the bacteria to accumulation of "excess" electrons which could be harmful to their regular activity and growth. One way by which this can be overcome is by converting the  $H^+$  ions to molecular hydrogen ( $H_2$ ). This is possible because of the specific hydrogenase enzyme they have developed, which catalyses the

simplified reaction:-



Most of the normal bacterial flora of the gastrointestinal tract use this reaction (Gray and Gest, 1965).

The exact process and the function of  $CH_4$  production is not clear. Its formation is confined to a specific group of bacteria - methanogenic bacteria - which require a strict anaerobic environment, and the production may possibly depend on the presence of  $H_2$  (Wolfe, 1971; Hungate, 1976; Sauer et al, 1977).

For a long time, the two gases  $H_2$  and  $CH_4$  have been known to be final products of anaerobic bacterial fermentation of organic substances. Their presence in the gastrointestinal tract of man has been suspected since Magendie (1816) identified  $CH_4$  in the intestinal gases in convicts recently guillotined. After this report, the source and even the production of  $H_2$  and  $CH_4$  in the gastrointestinal tract has been a subject of many controversies (Galley, 1954). Several workers since have measured  $H_2$  and  $CH_4$  from the gastrointestinal tracts of both ruminants and other animals (Mangold, 1934; Calloway, 1968).

In man, flatus has been extensively studied and the results have confirmed Magendie's findings (Kirk, 1949; Steggerda, 1968). The intestinal source of these gases was indisputably affirmed by intubating normal volunteers and measuring gas samples from different points along the gastrointestinal tract (Levitt and Ingelfinger, 1968). The bacterial origin of the two gases was demonstrated in vitro by culturing gut microflora with the appropriate substrates (Calloway et al, 1966; Gall, 1968), and in vivo by manipulation of gas

formation with antibiotics (Steggerda, 1968; Murphy and Calloway, 1972), or by the administration of substrates which are not digested by human gastrointestinal enzymes (Calloway and Murphy, 1968) (Fig. 1c). Mammalian cells neither need nor produce  $H_2$  or  $CH_4$  in their metabolism.

The behaviour of gas in the gastrointestinal tract has been of interest to many physiologists and a number of studies, in animals, were conducted to investigate the dynamics and factors which influenced it (Kantor, 1918; Hedin and Adachi, 1962; Forster, 1968; Gunbman and Williams, 1971; Murray et al, 1976). These investigations have led to the understanding that gases in the lumen of the gut are not in isolation from the rest of the body, but participate in the active process of exchange between the blood stream, body tissue and the external environment. McIver et al (1926), in an elegant experiment in the cat demonstrated the behaviour of intestinal gases and the factors which influence them. Under anaesthesia, they isolated measured segments of intestine and after cleaning the lumen, tied both ends without interfering with the blood supply. A known amount of <sup>the</sup>gases usually found in the gut (i.e.  $O_2$ ,  $N_2$ ,  $CO_2$ ,  $H_2$ ,  $CH_4$  and  $H_2S$ ), was then introduced into the ligated loop and the segment put back into the abdomen. At intervals the amount of gas remaining was analysed and measured. Different factors possibly affecting gas exchange, e.g. blood supply and relative concentrations of gases in the circulation, were manipulated and their effects recorded. From these detailed studies they were able to show that the movement of gases into and out of the intestinal lumen was governed by the usual laws of gas diffusion viz. difference in the partial pressure of the gases between the two sides, the thickness and area of the separating membrane and the diffusion

coefficient of the gas under consideration. They also found that  $\text{CO}_2$  had the highest absorption rate and  $\text{N}_2$  the lowest, and in decreasing order -  $\text{CO}_2 \rightarrow \text{H}_2\text{S} \rightarrow \text{O}_2 \rightarrow \text{H}_2 \rightarrow \text{CH}_4 \rightarrow \text{N}_2$ .

Despite the experimental confirmations of gas exchange between the intestinal lumen and blood circulation and the remarkably accurate measurement of breath  $\text{H}_2$  and  $\text{CH}_4$  by Parsons (1930) there appears to have been no active interest to utilise this method for assessing intestinal gas production. It is also of interest to note that the many reports of accidental fire and explosion, either spontaneously or during surgery using cautery, were thought to be rightly due to eructation of combustible gases from the stomach as a result of pyloric stenosis or gastrocolic fistula, with no attempt to implicate breath sources (Galley, 1954; Newman, 1974). With the development of gas chromatography in the fifties (Martin, 1969), it became relatively easy to measure the very small amount of  $\text{H}_2$  and  $\text{CH}_4$  usually excreted in the breath. Neilson (1961) is reported to be the first investigator to have developed the gas chromatographic method of  $\text{H}_2$  and  $\text{CH}_4$  measurement in the breath. During the mid sixties, Calloway and her group applied this method as part of investigation into the effects and role of intestinal gases in space flight and established most of the ground work (Calloway, 1968). Levitt and his group, systematically studied the site of  $\text{H}_2$  and  $\text{CH}_4$  formation, the behaviour of the two gases in the intestinal tract and the factors which influenced production and absorption (Levitt and Bond, 1970).

Some of the important findings by the above workers and others were:-

1. Almost all normal people produce some  $\text{H}_2$  in their gastrointestinal tract, while only a third of those studied produced significant amounts of  $\text{CH}_4$ .

2. Little or none of  $H_2$  is produced in the stomach and small intestine in normal circumstances while substantial amounts are formed in the colon. Methane is formed only in the colon.
3. External sources of nutrients, particularly nonabsorbable carbohydrates, act as substrates for the bacterial formation of  $H_2$ , while endogenous sources appear to have little influence on the level formed. Methane was unaffected by the few substrates tested.
4. The  $H_2$  and  $CH_4$  formed in the lumen of the colon are either absorbed into the blood stream and excreted by the lungs or are passed out of the anus as flatus.

The proportion of the two gases that is absorbed and excreted in the breath is not definitively known. McIver et al (1926) measuring the rate of absorption of injected  $H_2$  and  $CH_4$  from an isolated segment of small intestine of the cat, in situ, found 7.2ml of  $H_2$  (36%) and 4.3ml of  $CH_4$  (21.5%) absorbed in one hour, from a 25cm segment filled with 20ml of each gas separately. They also noted that the rate of absorption decreased as the relative concentration of the injected gas in the lumen decreased. Levitt and Ingelfinger (1968), after intubating human volunteers with triple lumen polyvinyl tubes and constantly infusing air containing a marker gas, measured gas samples from separate points with known distance and from the rectum and the breath. From their results they were able to calculate a 14% absorption for  $H_2$ . This figure is a gross underestimation of the actual amount capable of being absorbed if a constant washout method was not used. Calloway and Murphy (1968), on the other hand, from measurements of  $H_2$  and  $CH_4$  in the expired breath and flatus, under differing conditions, inferred that the two gases were mainly absorbed and excreted through the lungs. Their inference was

based on the following findings:

1. Little or none of the two gases were detected in flatus during ingestion of a Western type of diet or while fasting, though there was some being excreted in the breath.
2. A relatively high level of excretion of  $H_2$  in the breath after ingestion of gas-forming foods as compared to flatus.
3. The detection of  $H_2$  in flatus of some individuals only when they ingest a substantial amount of beans, though there was always  $H_2$  in the breath proportional to the amount eaten.
4. The findings by other investigators that when radiolabelled substrates were administered most of the activity was detected in the breath and not in flatus (Bernimolin et al, 1958).

Murray et al (1976) studying the rate of production of methane in the large intestine of sheep using radioactively labelled substrates showed that 89% of the  $CH_4$  produced is excreted through the lung.

Bond et al (1978), in their most recent animal study, have shown that  $CH_4$  is rapidly absorbed from the rat colon; half of the volume formed being absorbed in 90 minutes.

The absorption rate of the two gases was found to be greatest from the small intestine and colon and the least from the stomach and the rectum (McIver et al, 1926; Coburn, 1968). This may be due to the difference in the nature of the mucous membrane or in the blood supply. Physical laws which would be expected to control gas diffusion between the intestinal lumen and blood stream are:

1. The partial pressure gradient of the gas under the particular circumstance.
2. The nature, thickness and area of the separating membrane.
3. The solubility of the gas in the different media.
4. The molecular weight of the gas.



Since in the experimental situation, factors (1), (3) and (4) would be expected to be constant, the factor accounting for the difference must be the mucosa (i.e. factor 2). It is understandable for the function of the mucosal cells, the blood supply and the area per unit length to vary in the different parts, with the small intestine favourably disposed to absorption. The colon, rectum and stomach follow in that order. The experimental findings tally with this expectation.

For our purpose, it is important to identify the rate limiting factor for gas absorption from the colon. From theoretical considerations and available data, factors, 1, 3 and 4 are always in favour of  $H_2$  and  $CH_4$  diffusion into the blood stream, leaving factor 2, i.e. the mucosa, as the sole limiter. But, since the blood is continuously moving and being exposed to another gas exchange chamber at the lungs, blood flow would be expected to be another important variable. Coburn (1968) from his experiment on CO uptake from different parts of the gut, had calculated that more than 99% of  $H_2$  and  $CH_4$  uptake to be perfusion (i.e. blood flow) limited, implicating blood supply as the most important factor of the two. Though there is no available data on actual gut mucosal permeability to gases, one can infer from studies on gas absorption from the gut and other gas pockets in the body like pneumothorax, pneumoperitoneum or subcutaneous tissue, that gut mucosal permeability is high though not as high as the alveolar membrane. If this is the case and blood flow is the only rate limiting factor, another dimension to the picture arises and that is the role of the lungs. Since it is at the lungs that the two gases are cleared, it has been suggested that the actual limiting factor is the lung. But, the perfusion of the lungs is 3-5 times greater than that of the gastrointestinal

tract, and measurements on clearance rate of  $H_2$  and  $CH_4$  indicate that more than 80% is cleared during a single passage through the lungs (Calloway, 1968). So, blood flow to the gastrointestinal tract seems to be the only limiting factor of significance for the absorption of gases from the lumen.

Theoretically, 1g of carbohydrate completely fermented by bacteria would yield about 195ml of  $CO_2$ , 40ml of  $H_2$  and 75ml of  $CH_4$  (Calloway, 1968). At an excretion rate of about 4.0umol/l, 40ml of  $H_2$  would be excreted by the lungs in less than 2 h. In vivo situations, this amount of gas is not actually produced because of the partial nature of the anaerobic metabolism. Even if this amount of gas is produced, it is not produced instantaneously but over a period of time. Also, the capacity of the lungs to excrete  $H_2$  or  $CH_4$  is much greater than the level quoted above. Therefore, ingestion of a modest amount of dietary fibre would not be expected to overload the capacity for intestinal absorption so as to make the alternative excretion route through flatus necessary. Even if some  $H_2$  and  $CH_4$  are passed as flatus, there is ample evidence showing that the amount of the two gases excreted by the lungs to be proportional to their intestinal production (Calloway, 1968; Levitt, 1969; Levitt and Bond, 1970). Breath sampling, therefore, can be a useful and reliable method of gauging the colonic production of  $H_2$  and  $CH_4$ .

The central problem in sampling and detection of  $H_2$  and  $CH_4$  will be the very small amount (in parts per million) normally excreted in breath. Thus, the method of sampling has to withdraw a sufficient quantity for detection and should at the same time reliably reflect the concentration of the two gases in the alveolar capillary blood. Calloway and her group (1968) used a rebreathing



technique into multilaminated plastic bag. Levitt (1969) put his subjects inside a tight plastic hood, with  $O_2$  source and  $CO_2$  absorber plus an inert gas marker, and collected their breath over a period. Both these methods are uncomfortable for the subjects and cumbersome to use. A more versatile and equally reliable method of sampling was adapted and verified by Metz et al (1976a). The method involved the sampling of alveolar air by a modified Haldane-Priestley tube (Haldane and Priestley, 1905). Since then, an even simpler and portable method based again on the principle of Haldane-Priestley alveolar sampling has been devised.

All investigators using gas chromatography for measurement employed two separate detection systems for the two gases i.e. thermal conductivity (Katharometer) detector (K.D.) for  $H_2$  and flame ionization (F.I.D.) for  $CH_4$ . It is alleged that a katharometer detector is not sensitive enough for  $CH_4$  detection (Jeffrey and Kipping, 1964; Calloway and Murphy, 1968). It will be shown that this is not quite true in the methods section of this thesis.

In summary, the gases of  $H_2$  and  $CH_4$  are some of the final products of bacterial fermentation in the colon. These gases are mainly absorbed into the blood stream and excreted by the lungs. The level of excretion in the breath is proportional to the level of intestinal production of the two gases, making breath sampling and measurement of the concentration of  $H_2$  and  $CH_4$  a useful, non-invasive and simple method for the assessment and gauging of anaerobic bacterial fermentation in the colon.

### Conclusions

Dietary fibre is a complex material made up mainly of the polymers cellulose, hemicellulose, pectin and lignin. These polymers are not metabolised by human intestinal enzymes but are

fermented, to a degree, by colonic bacteria. The major metabolic byproducts of caecal fermentation of fibre are VFA and the gases  $\text{CO}_2$ ,  $\text{H}_2$  and  $\text{CH}_4$ . Dietary fibre because of its physical and chemical properties of absorption, adsorption, gel formation and cation exchange, influences the distribution and metabolism of a number of organic and non-organic substances of the gastrointestinal tract. As a consequence of these varied properties and its hydrolysis in the colon, fibre would be expected to have effects on a number of colon functions. At present, the fermentation of dietary fibre in the caecum and its effects on colon function are little understood.

There are indications that the hydrolysis of fibre and its effect might be influenced by the type and diversity of the colonic bacteria, and the chemical and physical properties of the component polymers, and the interaction of each polymer with the surrounding solute, bacteria and organic materials. The direct studies of these variables is hampered by the inaccessibility of the human colon, particularly the right side, where most of the fermentative, absorptive and secretory functions take place. Alternative methods of study are either animal experiments or indirect assessment of metabolic byproducts and some parameters of colon function. These may include the measurement of faecal wet and dry weight, faecal bile acids, electrolytes, fats and neutral steroids, and analysis of fibre polymers in the faeces, transit time assessment and the measurement of breath  $\text{H}_2$  and  $\text{CH}_4$ .

CHAPTER II

M A T E R I A L S      A N D      M E T H O D S

## MATERIALS AND METHODS

### A. Participants and Materials

Participants in the study were volunteers living in Edinburgh, aged 20 to 45 years. Most were members of staff in the hospital and the rest a group of physiology honours students. All were in good health at the time of participation.

Rats used were male Wistar strains supplied by the animal breeding centre of the University, and kept at the animal house of the Department of Biochemistry.

Most of the dietary fibre components used were obtained commercially. The bran was wheat bran characterised and supplied by the American Association of Cereal Chemists (AACC). The carrot, Flako Autumn King, was grown and supplied by Mr. J. Robertson, research student in our Unit. Detailed description of the dietary fibres and components used is attached as appendix (Appendix 1, 2 and 3).

### B. Breath Hydrogen ( $H_2$ ) and Methane ( $CH_4$ ) Measurement

#### Method of Sampling

The method adapted in this project is the modified method of Haldane and Priestley (1905) used by Metz et al (1976a) and is described in detail below.

The tube used for blowing into is made of polypropylene, 150cm long and 12mm internal diameter. To the proximal end is attached a 9cm glass mouth-piece. 18cm from the proximal end of the tube there is a hole into which is inserted a three-way-tap for connecting a 50ml syringe (polypropylene) for sampling air from the lumen of the tube (Fig. IIa).

The person whose breath is to be sampled sits in a chair and blows into the tube through a mouthpiece with one forced expiration

Figure - IIa

BREATH SAMPLING TECHNIQUE



following a normal inspiration. At the end of the forced expiration the mouth-piece is sealed with the tongue and then 50ml of expired air is withdrawn from the tube. This sample is a good approximation to an alveolar sample since it is from the last part of the forced expiration (Haldane and Priestley, 1905). The whole process takes less than one minute.

#### Storage of Samples

During a study session frequent breath samples are taken from several individuals. This results in accumulation of samples in syringes before analysis. Moreover, if the samples are not analysed immediately for any technical reason, they have to be stored for later analysis. To assess the rate of loss of the gases from the syringe, samples were stored for different durations of time and in variable conditions, and then analysed. Storage for about 7-10h at room temperature resulted in  $< 4\%$  loss for  $H_2$  and none for  $CH_4$ . Storage for 24h was followed by  $< 10\%$  loss for  $H_2$  and  $< 1.5\%$  for  $CH_4$ , 48h by  $< 17\%$  for  $H_2$  and  $< 2.5\%$  for  $CH_4$ . One week storage at room temperature brought about 50% loss for  $H_2$  and 20% for  $CH_4$ . Storage in the refrigerator at  $6^\circ C$  or at  $-20^\circ C$  had no effect on the rate of loss. The difference in the rate of loss of the two gases may be due to the difference in molecular size. In the studies reported in this thesis samples were analysed on the same day as the experiment and so the problem of leakage is insignificant.

#### Detection and Measurement

The analytical instruments used were a gas-solid chromatograph (GSC) (Pye Unicam, Series 104) with a katharometer detector (KD) and 1 mv pen recorder for display of values (Philips, PM8000). The chromatograph has a column and a detector chamber kept at constant temperature. In the column chamber are two glass columns

of equal size packed with a suitable stationary phase. In the detector chamber is the katharometer which use tungsten filaments arranged in a bridge circuit. Carrier gas from a cylinder passes at a defined pressure and flow rate, first through a purifying tube and then through both columns, followed by the detector chamber and out through the outlet tubes. One of the columns has a sampling loop with a control valve system attached in series. A modified tube containing soda lime and silica gel is connected to the sampling loop from the other side. A separate unit carries all the electrical control system (Fig. IIb).

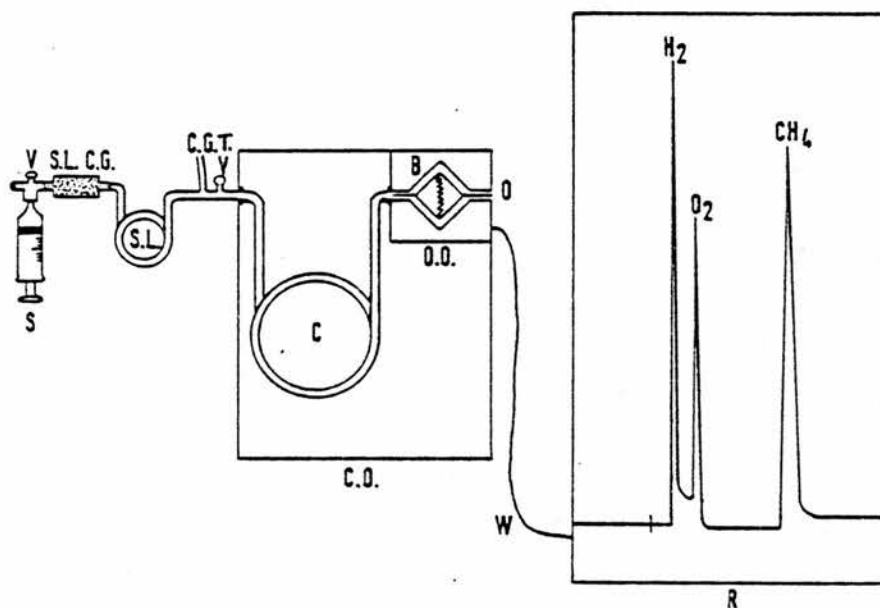
In the gas-chromatographic detection of  $H_2$  and  $CH_4$ , two different types of detectors are in general use, i.e. thermal conductivity (KD) for  $H_2$  and flame ionization for  $CH_4$  (Calloway and Murphy, 1968). This is reputed to be due to difference in sensitivity of the two systems for the two gases. To minimise cost and to simplify work the possibility of measuring the two gases simultaneously from one breath sample was examined using a thermal conductivity detector. Starting with the manufacturers' suggested working conditions for the chromatograph, the effect of changing each variable (e.g. detector temperature, bridge current, carrier gas, etc.) whilst keeping the others constant, on the sensitivity, peak resolution and retention time were assessed.

The results of the above mentioned exercises were essentially the following:-

When Argon (Ar) was used as carrier gas, the  $CH_4$  peak was obliterated by the comparatively huge  $N_2$  peak. This huge  $N_2$  peak was eliminated by using oxygen free  $N_2$  as carrier gas with good resolution of the  $CH_4$  peak. Though the sensitivity was decreased by the usage of  $N_2$  as carrier, the peak sizes were sufficient to

Figure - IIb

## DIAGRAM OF THE GAS SOLID CHROMATOGRAPH

Legend

(S)	Sample Syringe	(C)	Column
(V)	Valve	(D.O.)	Detector Oven
(S.L.)	Soda Lime	(B)	Bridge Circuit of filaments
(C.G.)	Cilica Gel	(O)	Outlet
(S.L.)	Sampling Loop	(W)	Electrical wire
(C.G.T.)	Carrier Gas Tubes	(R)	Recorder
(C.O.)	Column Oven		

The chromatograph represents 4.5umol/l standard gas



allow measurement (4.5  $\mu\text{mol/l}$  gave about 25% excursion on the chart) and were later improved by manipulating other variables.

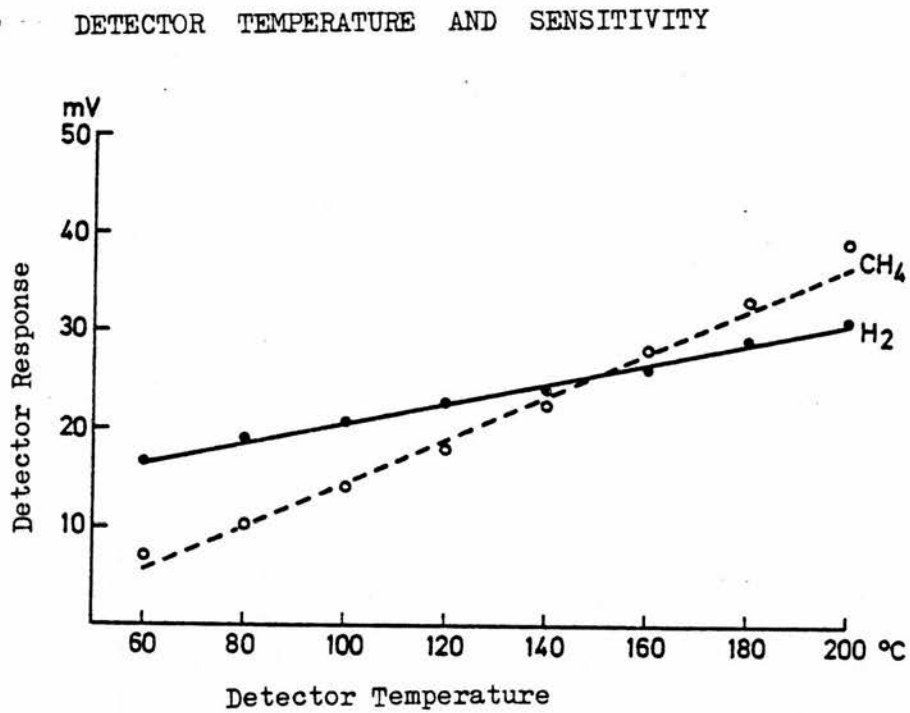
The sizes of the peaks were found to be proportional to the sampling loop volume and as such the biggest available size (10ml) was chosen. Column size affected the retention time, and as a result peak separation. Changing the mesh size of the stationary phase from coarse (30/65) to a finer one (60/85) improved the separation of  $\text{O}_2$  and  $\text{H}_2$  peaks. The effect of flow rate was mainly to change the retention time of each gas (i.e. faster flow rate, shorter retention time and vice versa), and to some extent separation of peaks and sensitivity of the katharometer. Direct clearance by the soda lime and silica gel of  $\text{CO}_2$  and water vapour from the breath sample improved base line noise and the life of the stationary phase.

The most important observations from altering column temperature, detector temperature and bridge current, on the sensitivity of the katharometer to the two gases were:

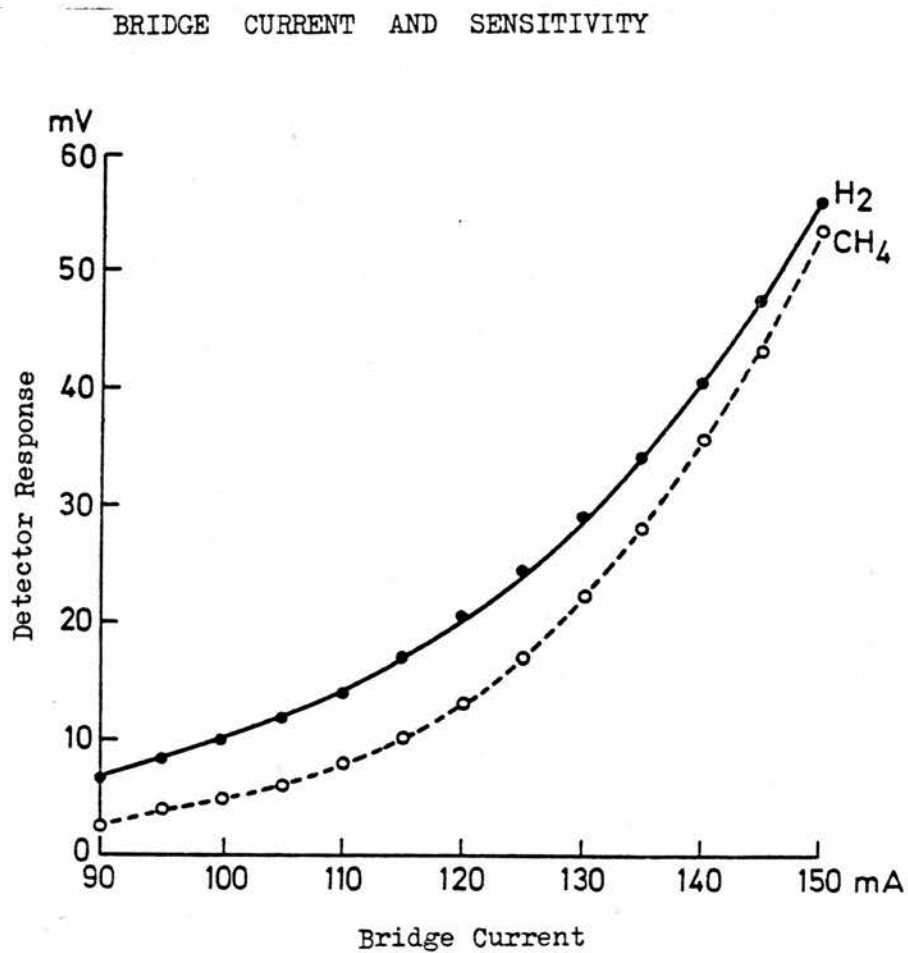
- (1) An increase in detector temperature increased the sensitivity to both gases, but more markedly for  $\text{CH}_4$  (Fig. IIc).
- (2) An increase in bridge current increased the sensitivity to both gases (Fig. IId).

These findings were important in that they were utilised to improve the sensitivity of the katharometer and compensate for the loss encountered by the use of  $\text{N}_2$  as carrier.

The above studies enabled suitable working conditions, within the limits of the gas-chromatograph, to be chosen (Table IIa). Fig. IIe is a chromatogram of 4.5  $\mu\text{mol/l}$  standard gas and a sample of breath from a person with a high  $\text{H}_2$  and  $\text{CH}_4$ , using these conditions. The first peak is  $\text{H}_2$  with a retention time of 65s, the second one  $\text{O}_2$  with

Figure - IIc

10 ml of 4.5  $\mu$ mol/l standard gas was injected at different detector oven temperatures all other conditions of the GSC being the same.

Figure - IIId

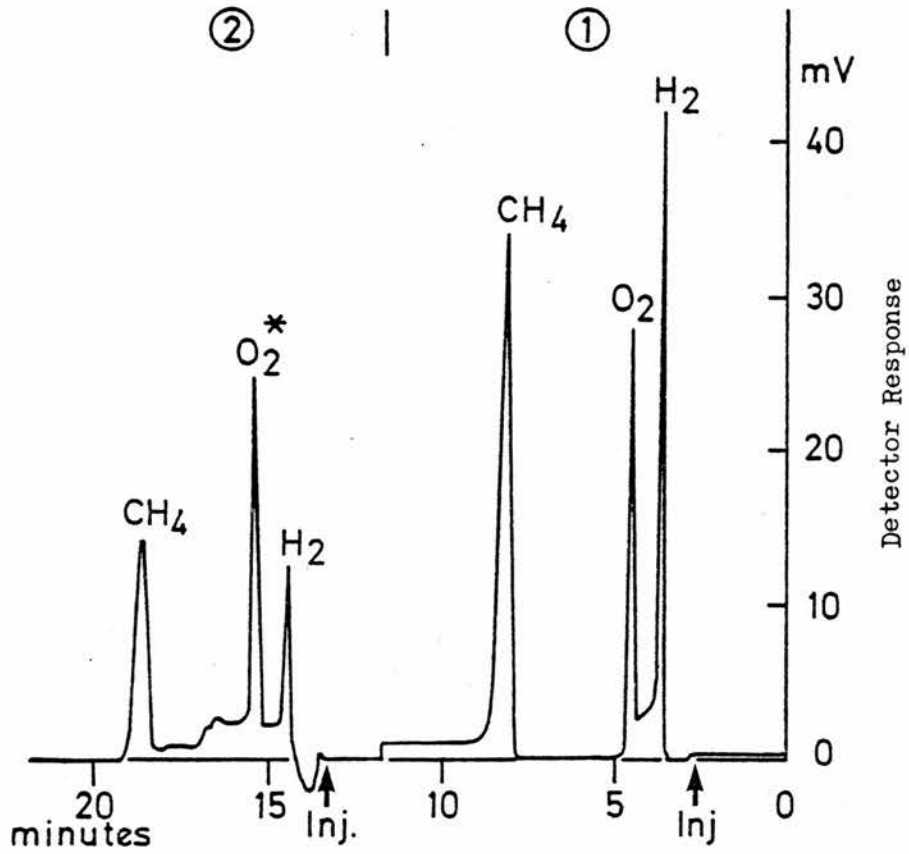
10 ml of 4.5  $\mu$ mol/l standard gas was injected at different detector filaments current, while all other conditions of the GSC were kept the same.

**Table - Ila****Chromatographic working conditions**

	<b>H<sub>2</sub> &amp; CH<sub>4</sub></b>	<b>Faecal Bile Acids (Ketones)</b>	<b>Rat Bile Acids (Methyl acetates)</b>
<u>Column</u>			
Type	Glass (silanised)	Glass (silanised)	Glass (silanised)
Size	2.6m (4mm internal diameter)	1.5m (4mm internal diameter)	1.5m (4mm internal diameter)
Pack (Type)	Molecular sieve - 5a	3/6 W - 17	3/6 S.E. - 30
Pack (Mesh)	60/85	100/120 (Gaschrom Q)	100/120 (Sampleport)
Temperature	50°C	270°C	262°C
<u>Detector</u>			
Type	Katharometer	Flame Ionization	Flame Ionization
Temperature	100°C	270°C	262°C
<u>Carrier Gas</u>			
Type	Oxygen Free Nitrogen (OFN)	Oxygen Free Nitrogen (OFN)	Oxygen Free Nitrogen (OFN)
Flow Rate	65ml/min	40ml/min	30ml/min
Pressure	202 kPa	206 kPa	206 kPa
<u>Sampling</u>			
Type	Loop (10 ml)	Direct Injection	Direct Injection
Temperature	Room temperature	290°C	280°C
<u>Attenuation</u>	1/2 Max. sensitivity	1/2 Max. sensitivity	1/2 Max. sensitivity
<u>Display</u>	Pen Recorder	Pen Recorder	Pen Recorder
<u>Chart Speed</u>	5mm/min	5mm/min	5mm/min

Figure - IIe

## CHROMATOGRAM OF GAS SAMPLES



## Chromatograms

- (1) 4.5  $\mu\text{mol/l}$  standard gas
- (2) Breath sample

See working condition of GSC from Table IIa

\* Attenuation set at 50 (x20)

Inj = Injection of Sample

retention time of 103s and the third  $\text{CH}_4$  with a retention time of 300s. Peak resolution between  $\text{H}_2$  and  $\text{O}_2$  is 3.3 and that between  $\text{O}_2$  and  $\text{CH}_4$  is 6.0. Fig. II f shows a plot of different concentration of standard gas against deflection on the recorder in millivolts. The relationship is linear with a slope of 0.49 for  $\text{H}_2$  and 0.36 for  $\text{CH}_4$ . This graph was used as a calibration of the instrument.

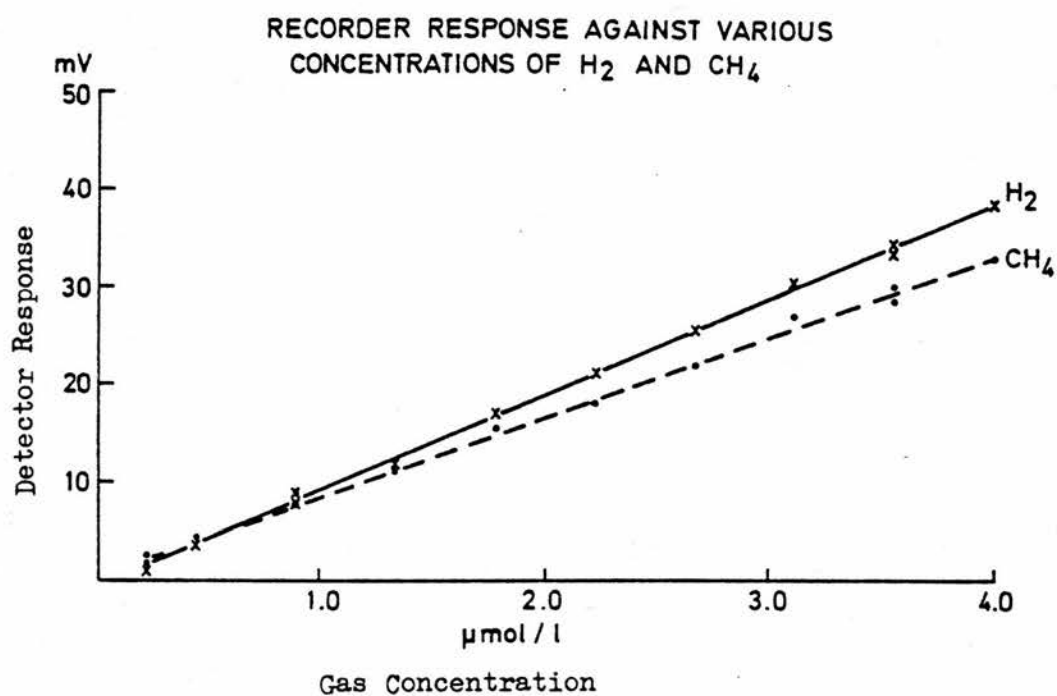
Reproducibility of results was tested in two ways. One, the deflection for 20 consecutive injections of 4.5  $\mu\text{mol/l}$  samples of the standard gases were measured. This showed a coefficient of variation of less than 4%. Second, a series of 30 pairs of breath samples, with a range of concentration of gases were analysed. The coefficient of variation for this series of tests was 1% for  $\text{H}_2$  and 7% for  $\text{CH}_4$ .

#### Smoking and Breath Sampling

While working with this method it was realised that smoking during sampling period would introduce an error in the  $\text{H}_2$  and  $\text{CH}_4$  level. Not being aware of this fact, five smokers who inhaled were investigated.

After one hour nonsmoking period, three base-line end-expiratory breath samples were taken at 3 minute intervals. Then each subject's choice of cigarette brand was roughly marked into three equal parts and the subject asked to smoke. The volunteers were interrupted and breath samples collected at the end of each smoked cigarette portion. After finishing the cigarette, samples were taken at 3, 6, 9 and 15 minutes post smoking.

Results for each individual are shown in Tables II b and II c, and the mean values plotted in Fig. II g. Sampling during a session of smoking will introduce an error in the  $\text{H}_2$  and  $\text{CH}_4$  values which

Figure - II f

10 ml of standard gas with known concentrations of  $H_2$  and  $CH_4$  were injected and recorded. See Table IIa for GSC conditions.

Table - IIb

End-Expiratory Breath H<sub>2</sub> Excretion (umol/l)

SUBJECT	PRE*			SMOKING**			POST*			
	0	3	6	1st	2nd	3rd	3	6	9	15
A	0.05	0	0	2.87	2.09	2.64	0.09	0	0	0
B	0	0	0.09	7.28	5.00	3.05	0.20	0.05	0.05	0.09
C	0	0.05	0	1.16	0.55	0.27	0	0	0	0.09
D	0	0	0	0.46	2.46	0.36	0	0	0	0
E	0.18	1.14	0.14	1.18	-	1.37	0.09	0.14	0.04	0.09
Mean	0.05	0.04	0.05	2.59	2.53	1.54	0.08	0.04	0.04	0.05
S.D.	0.08	0.06	0.07	2.77	1.85	1.28	0.08	0.06	0.06	0.05

\* Numbers indicate minutes

\*\* Numbers indicate end of portion smoked



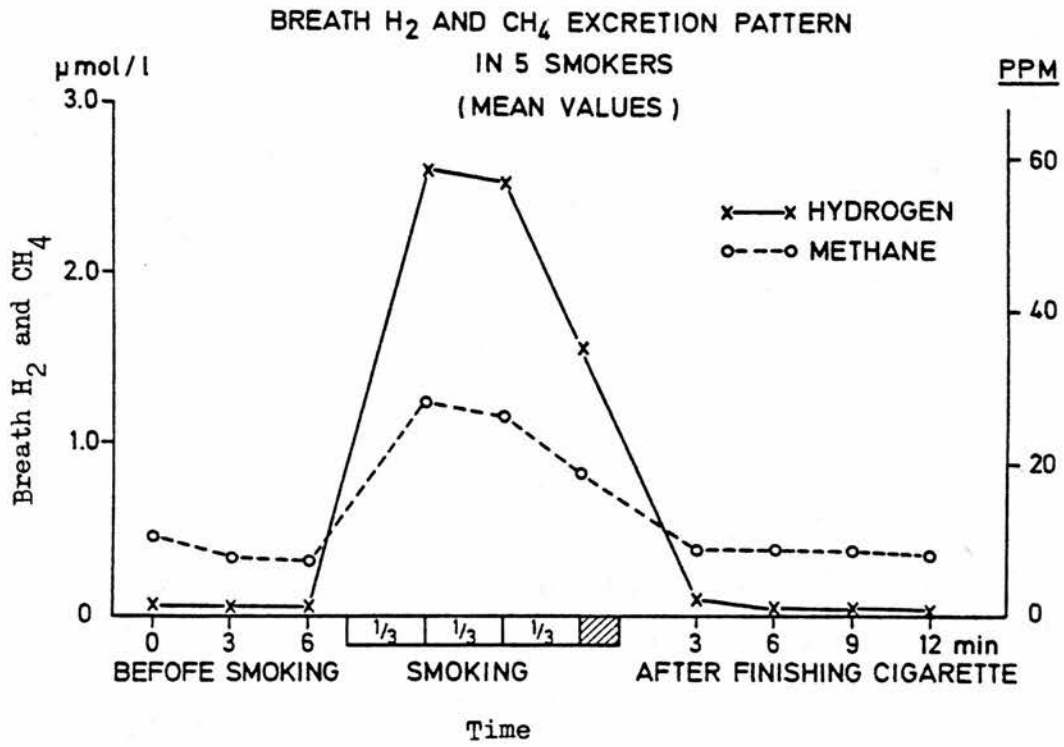
Table - IIc

End-Expiratory Breath CH<sub>4</sub> Excretion (umol/l)

SUBJECT	PRE*			SMOKING**			POST*			
	0	3	6	1st	2nd	3rd	3	6	9	15
A	2.57	2.00	1.89	4.00	3.71	3.71	2.78	2.57	2.66	2.64
B	0.09	0.09	0.14	3.40	2.05	1.37	0.05	0	0	0.05
C	0.18	0.09	0	0.91	0.36	0.18	0	0.14	0	0.05
D	0.05	0.05	0.05	0.32	0.68	0.18	0.05	0.09	0.09	0
E	0.32	0.14	0.18	0.41	-	0.50	0.05	0.05	0.05	0
Mean	0.64	0.47	0.45	1.81	1.69	1.19	0.59	0.57	0.56	0.55
S.D.	1.08	0.85	0.81	1.76	1.53	1.49	1.23	1.12	1.18	1.17

\* Numbers indicate minutes

\*\* Numbers indicate end of portion smoked

Figure - IIg

wears off quickly, presumably as the smoke is washed out of the lung. Being a cigarette smoker per se does not seem to alter  $H_2$  and  $CH_4$  excretion, but breath samples taken within moments of smoking could give a false-positive value as a result of contamination with the smoke.

That the source of error is the smoke and not the effect of smoking on  $H_2$  and  $CH_4$  production and/or excretion is supported by, (1) the fact that the pattern of  $H_2$  and  $CH_4$  excretion in the breath of smokers (when not smoking) is the same as that of nonsmokers, (2) the contaminatory effect of the smoke disappears rapidly and (3) direct analysis of cigarette smoke gives a very high level of the two gases.

#### Measurement of Bile Acids

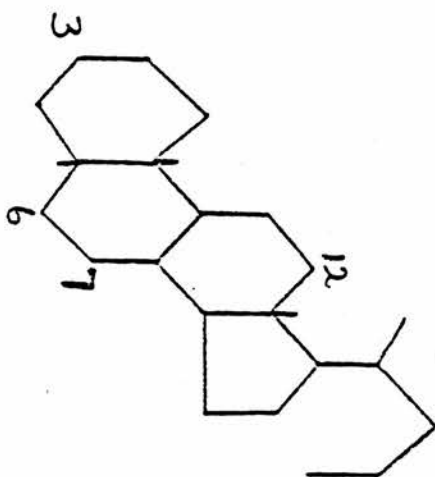
##### Measurement in the human faeces

The primary bile acids in man are cholic acid (3a, 7a, 12a-trihydroxy-5  $\beta$  -cholanoic acid) and chenodeoxycholic acid (3a, 7a-dihydroxy-5  $\beta$  -cholanoic acid). The main secondary bile acids are deoxycholic acid (3a, 12a-dihydroxy-5  $\beta$  -cholanoic acid) and lithocholic acid (3a-hydroxy-5  $\beta$  -cholanoic acid) (Fig. IIh). These acidic steroids or chemical products of them could be measured in a sample of dried faecal matter after repeated extraction and purification. The cyclic steroid nucleus is very resistant to oxidation and hydrolysis. This property is utilised in the extraction process and in the separation and identification of each bile acid. The method used in our laboratory, is based on the method of Evrard and Janssen (1969), and briefly involves the following steps.

Two tubes, one containing a known amount of freeze dried faeces with an internal standard 23-nordeoxycholic acid (1mg) and the other



Figure 4 Ith(1)



Primary Bile Acids

Cholic (C) 3a 7a 12a OH

Chenodeoxycholic (CDC) 3a 7a OH

Secondary Bile Acids

Deoxycholic (DC) 3a 12a OH

Lithocholic (LC) 3a OH

Muricholic Acids

$\alpha$  - Muricholic ( $\alpha$  MC) - 3a 6a 7a OH

$\beta$  - " ( $\beta$  MC) 3a 6a 7a OH

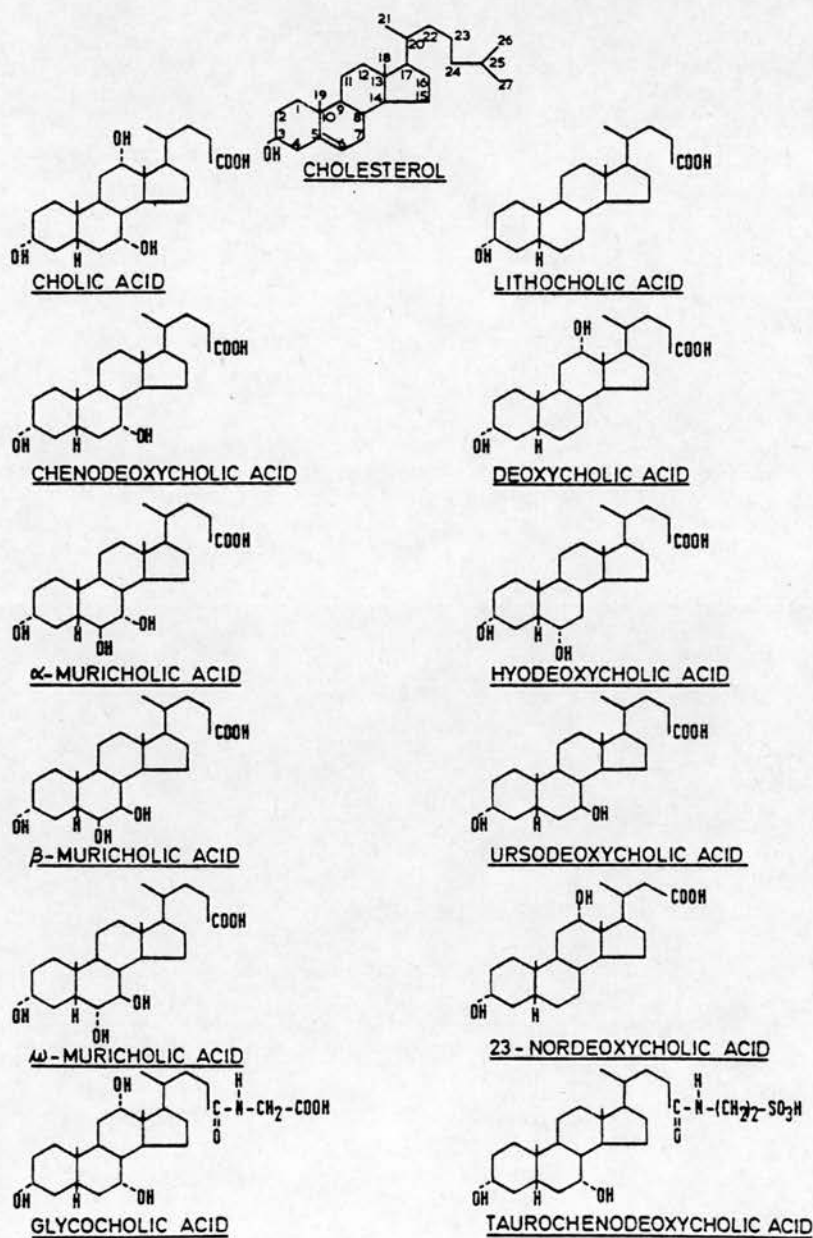
$\mu$  - " ( $\mu$  MC) 3a 6a 7a OH

Hydeoxycholic (HDC) 3a 6a OH

Ursodeoxycholic (UDC) 3a 7a OH

Figure - IIh(2)

## CHART OF BILE ACIDS



a mixture of reference bile acids (1mg of each) and the internal standard (1mg) are processed in duplicate. The bile acid moiety is extracted from the rest of the solid matter (protein, mucous, bile pigments, etc) by boiling with a toluene:acetic acid mixture (20:1, V/V). The conjugated bile acids in the extract are hydrolysed with 20% KOH in ethandiol and the neutral steroids removed by extracting with petroleum spirit. The free bile acids are then extracted by mixing several times with diethyl ether and then methylated using 2,2-dimethoxypropane. The methyl esters formed are converted to methyl ketones by oxidizing with chromic acid. A known amount of the ketone extract in acetone is injected into a gas-liquid chromatograph (Pye Series 104) with a flame ionization detector, for identification and quantitation. Detailed working conditions of the chromatograph are given in Table IIa and a typical trace in Figure IIIi. Individual bile acids are identified by a reference mixture or retention time and the amount determined by measuring peak height and comparing it to that of the internal standard in the sample and reference mixture, by using the following equation:-

$$M_x = \frac{S_x \times R_y}{S_y \times R_x} \times \frac{1}{W_s}$$

$M_x$  = amount in sample

$S_x$  = peak height of unknown in sample

$S_y$  = peak height of internal standard in sample

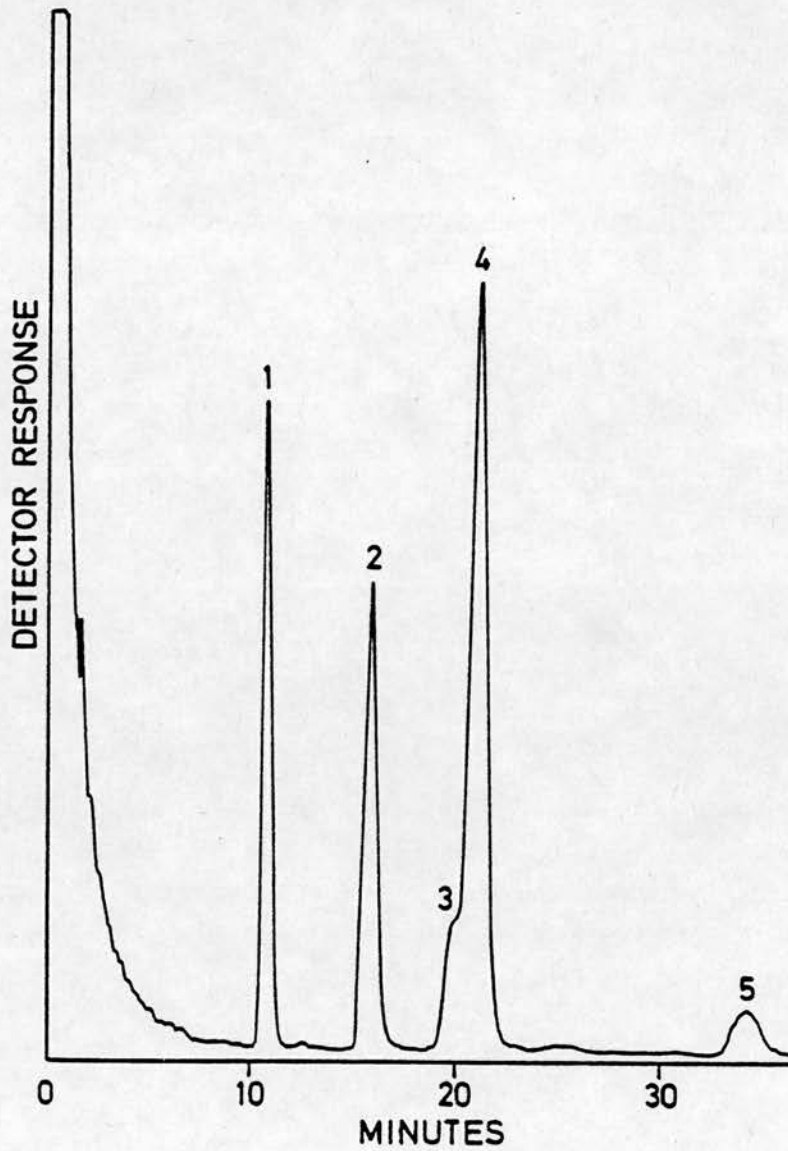
$R_x$  = peak height of known bile acid in standard mixture

$R_y$  = peak height of internal standard in standard mixture

$W_s$  = weight of faecal sample

Figure - IIIi

CHROMATOGRAM OF BILE ACIDS IN  
HUMAN FAECAL SAMPLES



Bile Acid Ketones: (1) NDC (2) LC (3) CDC (4) DC (5) C

See Table IIa for working conditions of GLC.



### Measurement in rat intestinal contents

In the rat, in addition to the above listed bile acids there are other specific bile acids known as muricholic acids (Fig. IIh):  $\alpha$ -muricholid acid (3a, 6 $\beta$ , 7 $\alpha$ -trihydroxy-5 $\beta$ -cholanoic acid),  $\beta$ -muricholic acid (3a, 6 $\beta$ , 7 $\beta$ -trihydroxy-5 $\beta$ -cholanoic acid) and  $\omega$ -muricholic acid (3a, 6a, 7 $\beta$ -trihydroxy-5 $\beta$ -cholanoic acid). Others in small amounts include ursodeoxycholic acid (3a, 7 $\beta$ -dihydroxy-5 $\beta$ -cholanoic acid) and hyodeoxycholic acid (3a, 6a-dihydroxy-5 $\beta$ -cholanoic acid). Because of the differences in the medium used and the bile acid pattern of the rat, the above described method needed some modification. First, the comparatively high concentration of fatty acids in the intestinal contents (particularly of the colon) hindered adequate extraction of the bile acids and required extra purification. Second, the presence of the hydroxyl group at C-6 of the muricholic acids close to that at C-7 disturbed the steric balance and led to breaking of the ring when oxidized and ketones formed; this made gas-chromatographic identification of the different muricholics difficult. These problems were overcome by combining the above method with that of Cohen (Cohen et al, 1975).

The procedure is essentially the same up to the end of the methylation stage. Then, some of the methyl ester extract is further purified by thin layer chromatography (TLC) using silica gel (MN-Silica Gel-G-HR) plate and developed by benzene followed by isooctane-isopropanolol-acetic acid (120:40:1, V/V/V). After spraying the plates with pyrene solution (Eastwood and Hamilton, 1968) bands are examined under ultra violet light. The bile acids, which are contained in the area between the origin to below the fatty acid band, are scraped from the plate and dissolved in acetone and filtered. Trimethylsilylether (TMS) derivatives are



then prepared using sylon HTP (Supelco-HMDA/TMCS and pyridine) and separated on GLC. This was used to identify the *m*-muricholic acid peak for which a reference sample was not available.

The remainder of the methyl ester extract is acetylated by adding 1ml from a cooled mixture of 14ml acetic acid, 10ml acetic anhydride and 1 drop of 70% perchloric acid (Roovers et al, 1968), and the methyl acetates extracted with diethyl ether after adding 20% NaCl. (TMS ether and methyl acetate derivative formation makes the steroid nucleus more stable and at the same time volatile for GLC separation).

Separation and quantitation of the methyl acetates is achieved on GLC (Pye Unicam, Series 104) with a flame ionization detector. See Table IIa for details of working conditions. Typical traces of methyl acetate derivatives of small intestinal and colon bile acids are shown in Figs. IIj and IIk.

#### Precision of Methods

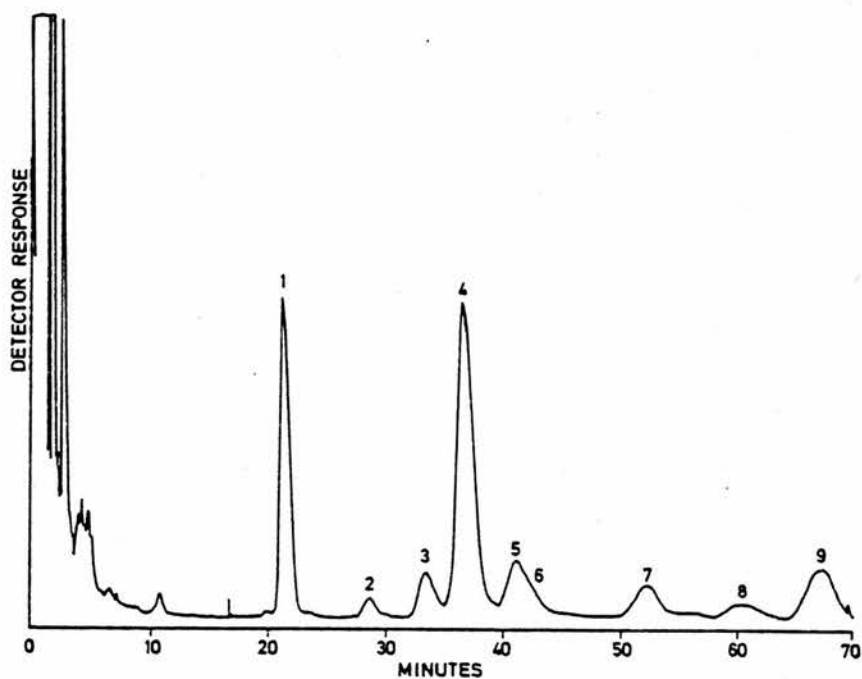
The recovery rate of the conjugated cholic was found to be 70-80% and that of the free bile acids 100%. The same faecal samples (n = 10) run on different days showed a coefficient of variations of 2.5% for the total and the following for each individual bile acids: cholic 3.5%, deoxycholic 3.4%, chenodeoxycholic 7.0%, and lithocholic 3.4%. A constant graph is kept in the laboratory to monitor drift.

For the rat intestinal contents recovery of added bile acids were as follows: lithocholic acid 98%; deoxycholic acid 100%; chenodeoxycholic 97%; hyodeoxycholic 100%; cholic acid 98%; Na taurocholate 77%; Na glycocholate 82%.

Processing of 10 pairs of small intestinal (range 16.0 - 57.6 umol) and colonic (range 9.0 - 62.6 umol) samples showed a coefficient of variation of 7.9% and 12.7% respectively.

Figure - IIj

CHROMATOGRAM OF BILE ACIDS  
IN RAT SMALL INTESTINE

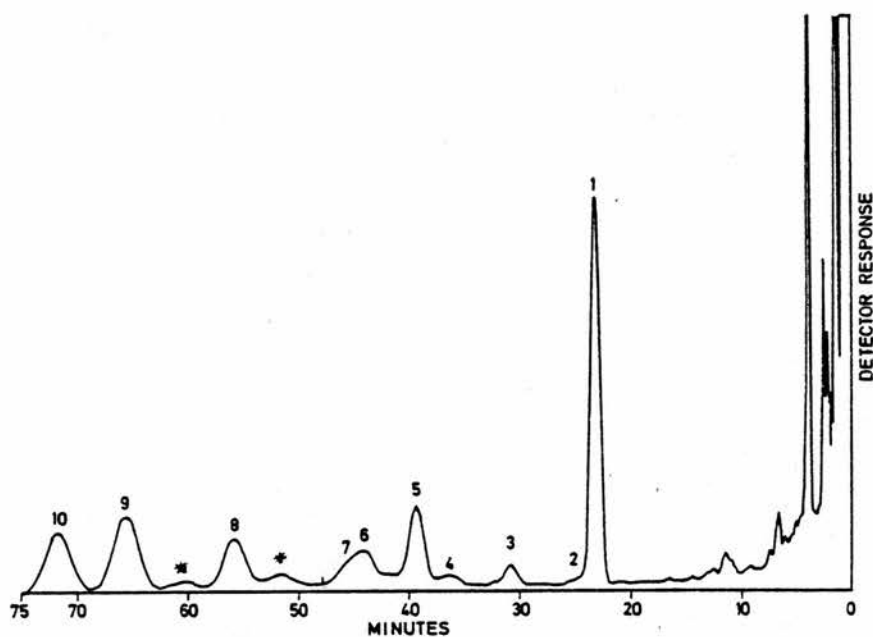


GLC separation of bile acid methyl ester acetates. (1) NDC (2) DC  
(3) CDC (4) C (5) HDC (6) UDC (7)  $\alpha$ -MC (8)  $\mu$ -MC (9)  $\beta$ -MC

See Table IIa for GLC working conditions.

Figure - IIk

CHROMATOGRAM OF BILE ACIDS  
IN RAT COLON



GLC separation of bile acid methyl ester acetates. See Table IIa for working conditions. (1) NCD (2) LC (3) DC (4) CDC (5) C (6) HDC (7) UDC (8)  $\alpha$ -MC (9)  $\mu$ -MC (10)  $\beta$ -MC.

\* unidentified peaks

Chemicals Used

Description of chemicals used in the different analysis is attached as Appendix (4).

CHAPTER III

P R E L I M I N A R Y      S T U D I E S

### PRELIMINARY STUDIES

In the experiments to follow, the breath  $H_2$  and  $CH_4$  is the principal measurement which has been used. This makes it necessary to determine the normal excretion pattern through the day of the two gases in the subjects studied. There is a lack of data on normal values for a Scottish population which makes such an exercise essential. Also, a detailed study of the normal pattern of excretion of these gases, it is hoped, would help in choosing appropriate conditions and timing for performing different diagnostic tests.

Another base-line measurement of importance to the study was the time it takes administered test meals to reach the colon. This was conveniently assessed by using the same method to be used as an indicator of dietary fibre metabolism in the colon, i.e. breath  $H_2$  test. Factors which possibly affect this mouth-to-caecum transit time (MCTT) were also looked into.

### 1. Normal Variation of Breath $H_2$ and $CH_4$

The breath  $H_2$  excretion in population with typical American or European diet has been shown to be of the order of 0.22 - 0.45  $\mu\text{mol/l}$ , with 0.91  $\mu\text{mol/l}$  as the accepted higher limit (Calloway, 1968).

Methane excretion is individual and follows its own cycle. About a third of the population are found to be  $CH_4$  excretors ( $> 1.0 \mu\text{mol/l}$ ) and the rest non-excretors ( $< 0.1 \mu\text{mol/l}$ ) (Levitt and Bond, 1970).

In this study besides determining the level of excretion we also examined the pattern of variation through the day and the factors which influence this pattern.

#### Method

Two population groups were studied. In the first group 10 males and 10 females aged 20 to 45 years were investigated:

1. While on their habitual diet
2. Fasting for about 20 hours

The test was conducted during their regular work routine.

Breath samples were taken at hourly intervals from 09.00 to 17.00 h. Some were sampled for a longer period extending over 10 hours. Half of the participants were studied on three consecutive days and the other half on three separate days in different weeks. For the fasting situation, subjects were fasted from 22.00 h to 15.00 h the following day. On the whole a total of 60 man-days of testing were performed unfasted and 20 man-days fasting.

A one week recall dietary history was taken by a dietician on 15 of the volunteers mainly to estimate their dietary fibre intake, but other dietary components were also estimated at the same time. The average daily nutritional intake was calculated by using a computer programme based on the food table by McCance and Widdowson (McCance and Widdowson, 1960). Small intestinal absorption status

was assessed by the D-xylose absorption test. Subjects were fasted overnight and at 09.00 h the following morning 5g D-xylose dissolved in 200 ml of water was administered by mouth. Participants were asked to empty their bladder before taking the test meal and urine collected over five hours thereafter. Within the first hour they took an additional 800 ml of water to make a total of one litre, but otherwise were fasted during the period of the test. D-xylose excretion in the urine was measured by using a colorimetric method (Varley, 1967). The five hours urine collection is first diluted to a litre and further 1 to 10 with water. 1 ml of the diluted urine and 5 ml p-bromoaniline reagent is added into two test tubes (blank and test). The tube containing the test sample is placed into a water bath at 70°C for 10 minutes. Then both the test tubes are left in the dark for 70 minutes. The pink colour that develops is then read by a spectrophotometer at 520 mμ against the blank. At the same time two standard solutions of xylose containing 0.2 and 0.1 mg/ml are processed as above and then read against their own blank. Using the standard containing 0.2 mg/ml the xylose excreted in the urine is calculated:

$$\text{Xylose excreted (g)} = \frac{\text{reading of unknown}}{\text{reading of standard}} \times 2$$

In 10 of the subjects in the above group, respiratory functions were assessed by using three variables:

1. Tidal Volume ( $V_T$ ) - was measured by a Benedict-Roth spirometer and the gas volume corrected to BTPS.
2. Respiratory Minute Volume ( $\dot{V}_E$ ) - breath was collected by means of a Douglas bag over 5 minutes and resting. Gas volume was measured by a gas meter, corrected to BTPS and



the volume per minute calculated. Results were compared with those calculated from the spirometer measurement.

3. Timed Vital Capacity ( $FEV_1$ ) - a vitalograph was employed and measurement done standing. A mean of three one second vital capacity was recorded.

The subjects age, weight and height were also noted.

The value for the concentration of  $H_2$  and  $CH_4$  in each breath sample was compiled for each subject and later plotted against the time of the day. These helped in comparing the excretion pattern of each volunteer on different sampling days, while fasting, and to the pattern of excretion of the other members of the group. The breath  $H_2$  values for each hour of the day from all the subjects were then pooled and the mean excretion for the hour and its standard error computed. The average excretion pattern during the day was drawn for the group by plotting the mean values against the time of the day.

For those subjects who had their  $\dot{V}_E$  measured, the daily breath  $H_2$  excretion was estimated by multiplying the mean  $H_2$  concentration per breath sample by their 24 h respiratory volume and the factor 0.80, the correlation coefficient between alveolar and mixed expiratory  $H_2$  concentration found by Metz et al (1976) when validating the sampling method used in this study (i.e.  $H_2 \times 0.80 \times \dot{V}_E \times 60 \times 24$ ). For the rest of the group, the mean  $H_2$  concentration per sample for the group and the  $\dot{V}_E$  value of 6 l/min was used.

The correlation between breath  $H_2$  and  $CH_4$  excretion and dietary fibre intake was calculated using the least-square linear regression.

In the second group, 68 volunteers (30 males and 38 females) aged between 45 and 90 years and living in the Pilton Area of Edinburgh were randomly selected from records in one general practice. A single end-expiratory breath sample was collected at their residence. Samples were taken in the morning and analysed on the same day. After measuring the  $H_2$  and  $CH_4$  in the samples

the mean excretion levels and the standard error was calculated for the group. These subjects with the rest of the volunteers tested were divided into three age groups: those who do not excrete  $\text{CH}_4$ , those who excrete 0.1 to 1.0  $\mu\text{mol/l}$  and those who excrete above 1.0  $\mu\text{mol/l}$  noted. The percentage of persons in each group was plotted in a histogram and compared. The relationship of  $\text{CH}_4$  excretion status to sex and also chronic disease state was looked into.

## RESULTS

### Group 1

General - Table IIIa is a summary of findings of the 10 volunteers who were studied in more detail. The D-xylose excretion and the respiratory function measurements were within normal limits. The mean 5 hours D-xylose urine excretion for the 15 subjects was 1.75g (range 1.5 - 2.1g) giving an average excretion of 35.2% (range 30-39%). The mean  $V_T$  for the 10 was 592 ml (range 500 - 760 ml),  $V_E$  - 6.6l/min (5.0 - 9.0 l/min ) and FEV - 90% (range 80 - 96%).

Non-fasting - Hydrogen excretion was found to have a regular pattern being comparatively high in the morning, falling about mid-day and rising again during the early afternoon (Fig. IIIa). Individual breath  $\text{H}_2$  excretion above 0.90  $\mu\text{mol/l}$  were rare and the mean at any time during the day was below 0.50  $\mu\text{mol/l}$ . The average daily excretion of  $\text{H}_2$  by an individual was calculated to be 68 ml (range 22 - 150 ml). In contrast  $\text{CH}_4$  excretion did not follow any regular pattern over the brief period studied and was found to be individual (Fig. IIIb). A third of the participants excreted above 0.10  $\mu\text{mol/l}$  (range 0.10 - 2.70  $\mu\text{mol/l}$ ) and the rest below 0.10  $\mu\text{mol/l}$ .

Measuring the breath  $\text{H}_2$  excretion of an individual on separate

Table IIIa

Different Datas for the Ten Subjects Studied

Subject	Sex	Age (yr)	Wt. (Kg)	Ht. (cm)	$V_T$ (ml)	$\dot{V}_E$ (l/min)	FEV <sub>1</sub> (%)	D-xylose Excretion (g) (g%)
1	M	20	65.5	176	610	7.5	80	1.5 30
2	M	20	65.7	178	590	7.5	93	1.6 32
3	F	21	50.5	159	640	5.5	96	1.8 36
4	F	21	65.3	169	510	5.5	93	1.8 36
5	M	20	67.1	184	570	9.0	95	1.5 30
6	M	21	64.9	172	500	5.0	94	1.8 36
7	F	21	63.0	176	580	5.5	96	1.9 38
8	F	20	63.5	164	660	5.5	82	1.7 34
9	M	20	69.9	176	760	8.0	88	1.8 36
10	F	26	51.7	164	500	7.0	83	1.9 38
Mean S.D.		21.0 1.8	62.7 6.4	171.8 7.7	592 82	6.6 1.4	90 6	1.7 35.4 0.2 2.7

Figure - IIIa

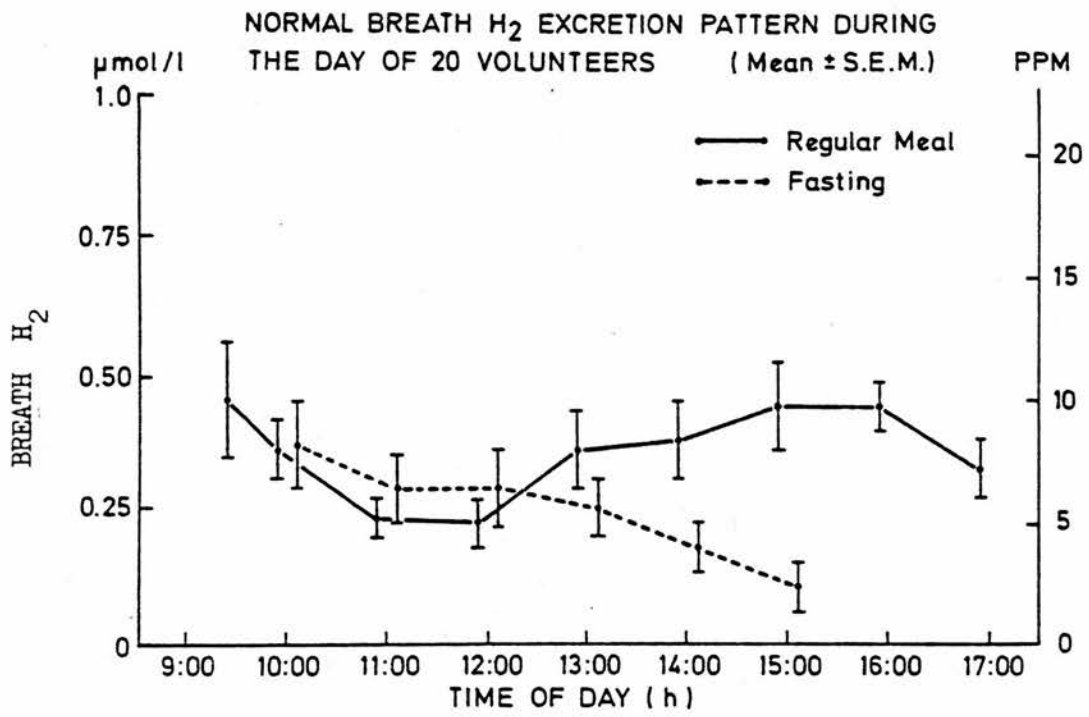
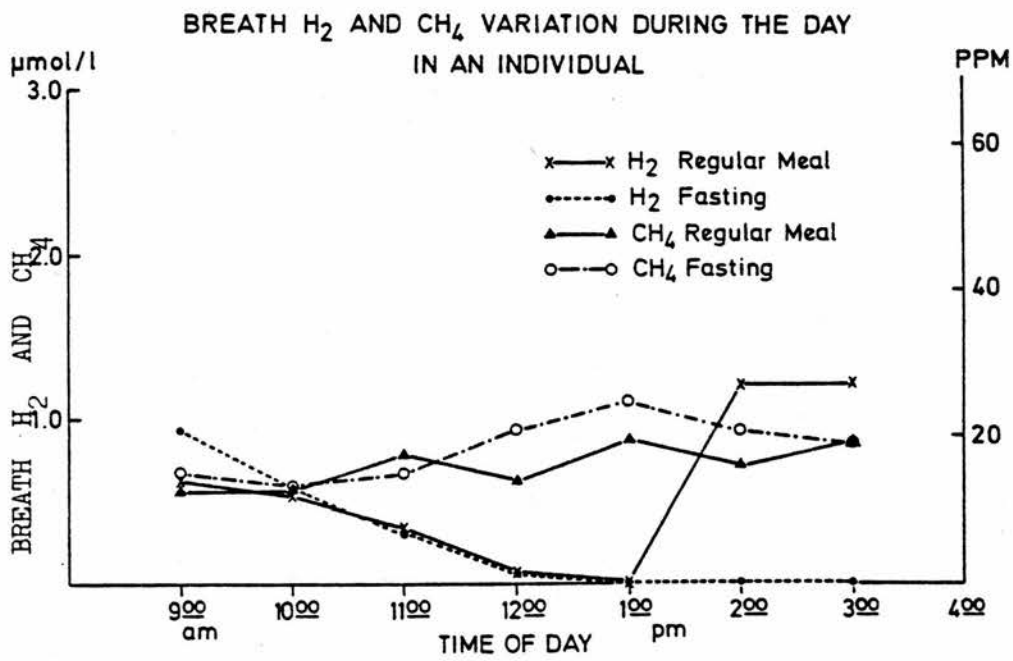


Figure - IIIb



days or on three consecutive days showed that the pattern of excretion remained essentially similar even though there was some slight variation in the actual amount excreted (Fig. IIIc). Fig. IIIId demonstrates the excretion pattern of  $H_2$  and  $CH_4$  for an individual over a 30 hour period interrupted by 7 hours sleep.

Table IIIb shows the calculated dietary intake of the 15 subjects. The mean dietary fibre intake was  $16.4 \pm 4.4g$  and there were no unusual findings for the other dietary constituents. There was no correlation between the estimated dietary fibre intake and the level of  $H_2$  or  $CH_4$  excretion ( $r = 0.18$  and  $r = 0.23$  respectively). The same applies to the protein, fat and carbohydrate intake.

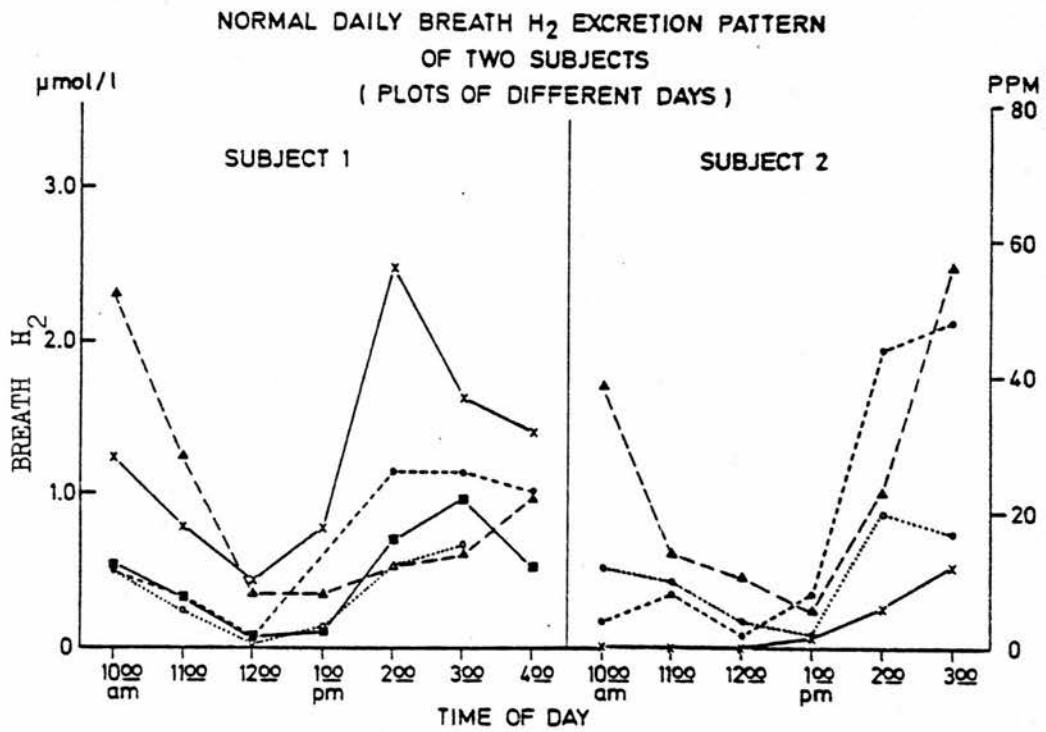
Fasting - Fasting for about 20 hours decreased the overall level of  $H_2$  excretion and abolished the afternoon rise. Excretion levels consistently went down during the day (Figs. IIIa,b). The average daily breath excretion of  $H_2$  in a fasting subject was calculated to be 28 ml (range 0 - 106 ml). Methane excretion was unaffected and remained individual (Fig. IIIb).

## Group 2

For the second group of volunteers the mean  $H_2$  excretion level was 0.25 umol/l (range 0 - 1.07 umol/l) and  $CH_4$  excretion 0.59 umol/l (range 0 - 4.45 umol/l). In marked contrast to the first group and reports from elsewhere, two-thirds of the group were  $CH_4$  excretors. There was no correlation of  $CH_4$  excretion status with sex or chronic disease state (e.g. diabetes, hypertension, chronic bronchitis, etc.) but the number of excretors increased with age (Fig. IIIe). Also excretion levels were higher in the older age group.

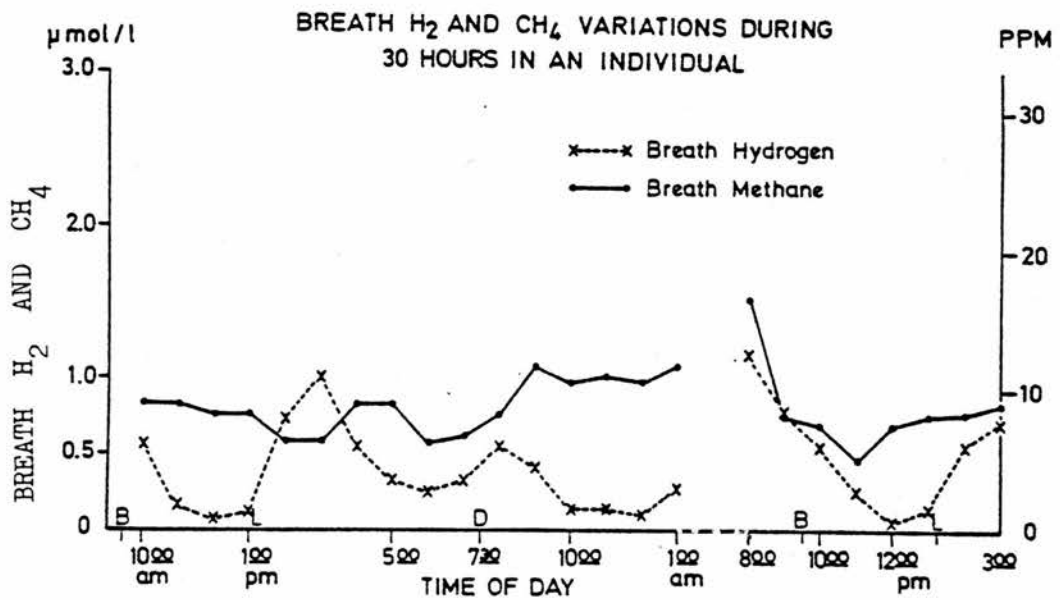
## Mouth-to-Caecum Transit Time (MCTT)

In the studies to follow, the plan consisted of administering dietary fibre or some chemical components of fibre in an acceptable

Figure - IIIc

Subject 1 - plots of five separate days in different weeks.

Subject 2 - plots of four consecutive days.

Figure - IIIId

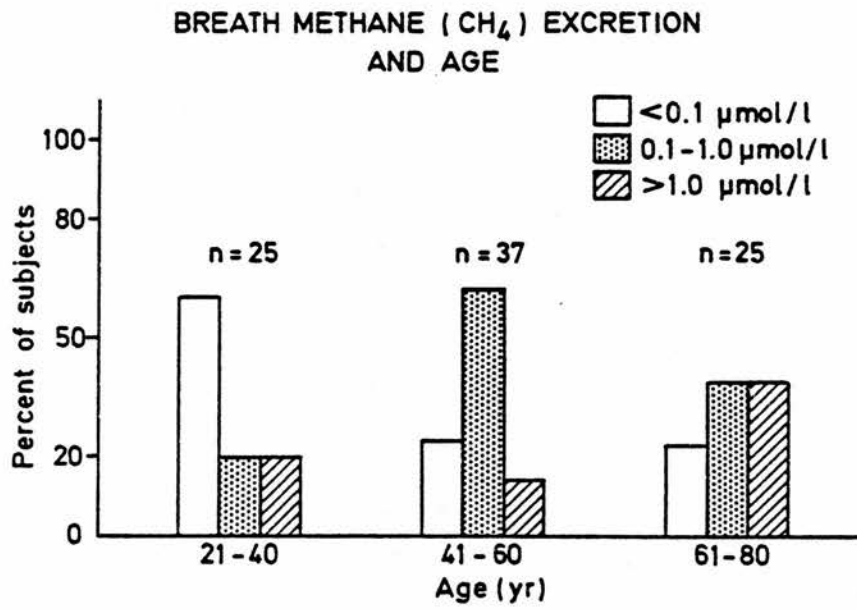
Plot of end-expiratory sample concentrations in an individual following regular daily activities and eating routines. Gap between 1 - 8 a.m. is sleep period. Breakfast (B), Lunch (L), Dinner (D).



Table - IIIb

Daily Dietary Intake and Transit Time Measurements

Subject	Protein (g)	Fat (g)	Carbohydrate (g)	D.F. (g)	S.S.T. (h)	M.C.T.T. (min) L* R*
1	64	90	228	9.8	68	70 -
2	67	102	262	20.0	36	- 120
3.	70	98	262	12.9	39	90 -
4	109	98	244	17.0	-	100 -
5	73	97	263	19.0	47	70 -
6	83	137	428	11.7	48	100 -
7	136	240	587	23.9	35	180 -
8	75	116	296	13.4	48	- 80
9	61	64	127	15.4	44	140 0
10	82	79	184	16.8	59	- 210
11	99	138	280	23.1	24	- 210
12	68	97	186	14.9	40	- 220
13	74	117	251	16.1	-	- 150
14	90	115	258	21.6	32	- 200
15	68	81	328	11.0	69	80 -
Mean	81	111	279	16.4	45	104 170
S.D.	20	41	109	4.4	14	38 54

Figure - IIIe

form by mouth and following the metabolism in the colon. Hence, it is important to have an idea of how long a fibre source test meal takes to reach the colon and the factors that influence this time.

There are several techniques for measurement of gastrointestinal transit time (i.e. the time taken by ingested substance to cover a certain distance of the gut). The principle is to administer a non-absorbable marker by mouth and identifying it at a certain distance of the gastrointestinal tract directly (sampling, X-ray, etc.) or indirectly (telemetry, radioactivity, etc.). The time taken from administration to detection gives the transit time. Coloured glass beads, carmine, seeds, ball bearings, barium, radioisotopes, radio-opaque polyethelene pellets and radiotelemetering capsules are some of the substances that have been used. Breath  $H_2$  measurement has also been used to assess MCTT or small intestinal transit time (SITT) (Bond and Levitt, 1975). Since in this study breath  $H_2$  measurement is being used as one of the parameters for dietary fibre metabolism the other application of the method is utilised to measure MCTT. The principle here is to give a non-absorbable oligosaccharide (e.g. lactulose) dissolved in water and then measure the breath  $H_2$  excretion at frequent intervals. The time from ingestion of the test meal to the first significant increase of breath  $H_2$  is the MCTT.

#### METHOD

Participants in the study were 15 volunteers (7 female and 8 males) free of clinical problems and aged between 21 to 31 years.

The oligosaccharides studied were the disaccharide lactulose, the trisaccharide raffinose and the tetrasaccharide stachyose. Ten grams of the oligosaccharide was administered dissolved in 150 or 300 ml water. End-expiratory breath samples were taken every 10 minutes as described in the methods section.

The participants were randomly divided into two groups and either lactulose or raffinose 10g in 150 ml water administered without fasting. A group of four participants took both oligosaccharides at different osmolalities (Table IIIc), sequentially on different days after fasting. Two in the last group in addition took stachyose. An approximate estimation of the viscosity of the solutions was done by a modified Ostwald viscometer.

The MCTT was calculated as the time for the first increase of breath  $H_2$  from base line concentrations (Bond and Levitt, 1975) and also when it exceeded 0.90  $\mu\text{mol/l}$ , which is the accepted upper limit of normal breath  $H_2$  excretion (Metz et al, 1976b).

On a separate week, the participants had their whole gut transit time measured with barium impregnated markers using single stool transit time (Cummings and Wiggins, 1976). Three different shapes (i.e. cubes, circles and cylinders) of radio-opaque polyethelene pellets of approximately the same specific gravity and similar behaviour in the gut were used. Twenty of the specified shape of markers for the particular day were administered with breakfast on three successive days and the first stool on the fourth day collected. The time of administration of each marker and the collection of the stool is noted. After freezing, the stool is X-rayed (Fig. IIIf) and the different markers counted. The transit time is calculated by using the formula:-

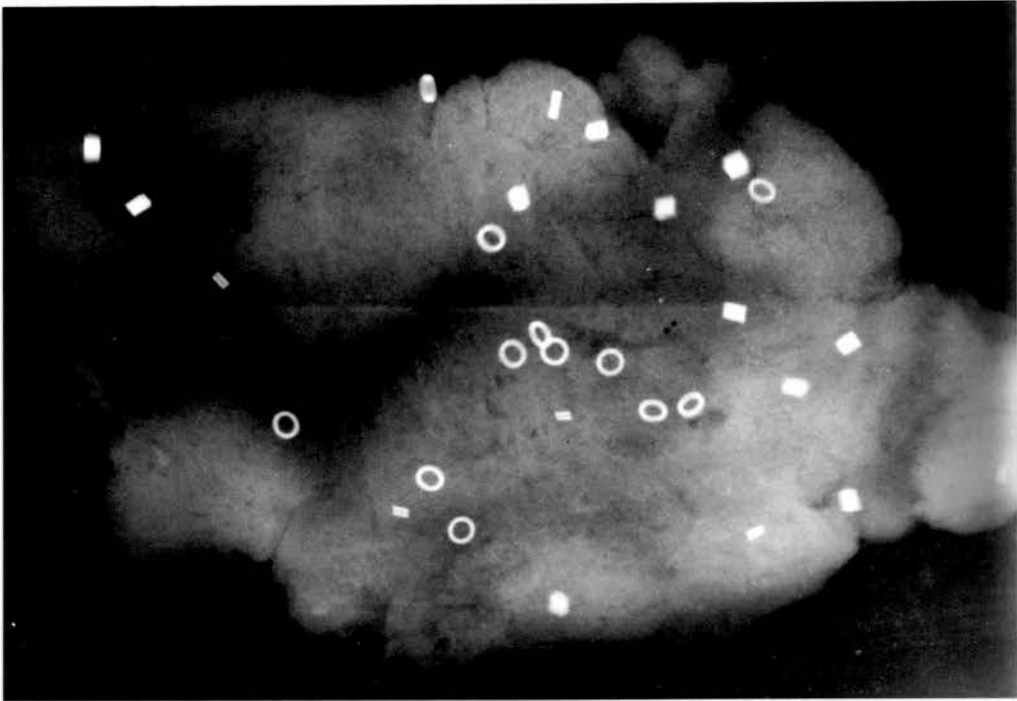
$$\text{SST} = \frac{t_1 s_1 + t_2 s_2}{s_1 + s_2}$$

SST = single stool transit time

$t_1$  &  $t_2$  = time from ingestion of marker to time of stool collection (number subscripts denote the two marker types in greatest number in the stool).

Figure - IIIf

RADIOGRAM OF STOOL WITH TRANSIT MARKERS



Stool radiogram from a fourth day collection after administration of 20 markers on three consecutive days. Cubes - first day, cylinder - second day and circle - third day.

$s_1$  &  $s_2$  = number of markers in the stool (number subscripts as above).

Dietary fibre intake was assessed and calculated by a dietician from a one week recall dietary history as previously described (McCance and Widdowson, 1960).

## RESULTS

The mean overall lactulose MCTT was 104 min (range 70 - 180 min); the single stool transit time 45 h (range 35 - 69 h) and dietary fibre intake 16.4 g (range 9.8 - 23.9 g). Within the accuracy limits of the method employed the viscosity of the different oligosaccharide solutions were not significantly different from that of water. For each of the oligosaccharide solution administered reducing the osmolality by half did not have significant effect on MCTT. Neither did fasting and non-fasting states (Table IIIId). However, changing the oligosaccharide molecular weight altered the transit time, with the larger molecular weight oligosaccharide having the longer transit time. The representative plot of the breath  $H_2$  changes against time for the three oligosaccharides in one fasting individual in Fig. IIIIf illustrates this point. The mean fasting MCTT for the three oligosaccharides studied (when breath  $H_2$  is 0.90  $\mu\text{mol/l}$ ) were: lactulose  $90 \pm 7$  min, raffinose  $168 \pm 35$  min, stachyose 290 min (Table IIIId).

There was no significant correlation between MCTT and SST ( $r = -0.52$ ); neither between MCTT and estimated dietary fibre intake ( $r = -0.59$ ). However, there was a highly significant ( $p < 0.001$ ) negative correlation ( $r = -0.76$ ) between single stool transit time and dietary fibre intake.

Breath  $\text{GH}_4$  was unaffected by any of the three oligosaccharides given and remained individual.

Table IIIc

Mouth-to-Caecum Transit Time (min) of Different

Oligosaccharide Solutions Measured by Breath  $H_2$ (when  $H_2 > 0.9 \text{ umols/l}$ )

Subjects **	Lactulose (470)	Lactulose (205)	Lactulose (550)	Lactulose* (215)	Raffinose (125)	Raffinose (60)	Stachyose (215)
A	80	90	100	-	150	160	290
B	110	70	-	100	200	200	290
C	90	90	-	-	240	260	-
D	80	70	70	90	80	70	-
Mean	90	80	85	95	168	173	290
S.E.M.	7	6	15	5	35	40	0

\* pure crystalline Lactulose

\*\* numbers in bracket indicate osmolalities in mmol/kg



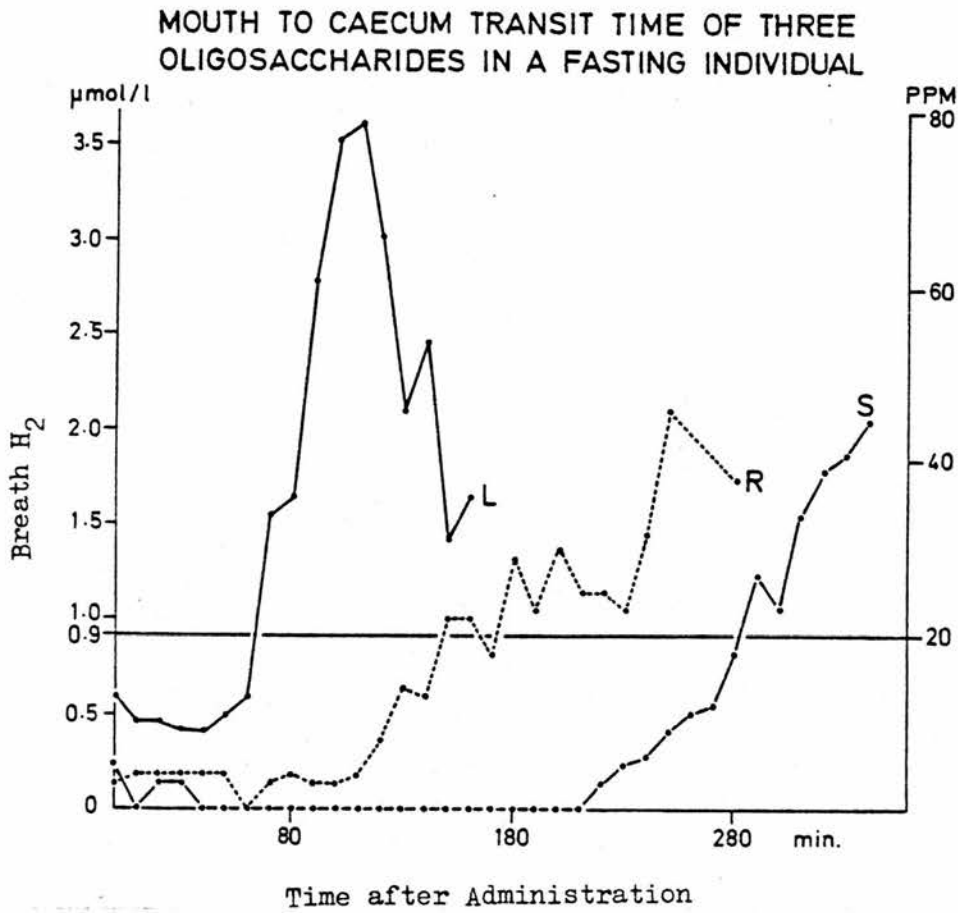
Table - IIIdMouth-to-Caecum Transit TimeMeasured by Breath H<sub>2</sub>(Mean  $\pm$  S.E.M.)

Substance	Non-fasting		Fasting	
	Initial Rise (min)	> 0.9 $\mu$ mol/l (min)	Initial Rise (min)	> 0.9 $\mu$ mol/l (min)
<u>Lactulose</u>	(10)		(4)	
470 mmol/kg	78 $\pm$ 7	104 $\pm$ 13 <sup>1</sup>	66 $\pm$ 2	90 $\pm$ 7 <sup>3</sup>
205 mmol/kg	—	—	63 $\pm$ 5	80 $\pm$ 6
<u>Raffinose</u>	(9)		(4)	
125 mmol/kg	128 $\pm$ 18	170 $\pm$ 20 <sup>2</sup>	115 $\pm$ 33	168 $\pm$ 35 <sup>4</sup>
60 mmol/kg	—	—	90 $\pm$ 19	173 $\pm$ 70
<u>Stachyose</u>			(2)	
215 mmol/kg	—	—	185 $\pm$ 35	290 $\pm$ 0 <sup>5</sup>

1,2 -  $p < 0.02$ 3,4 -  $p < 0.05$ 4,5 -  $p < 0.05$ 

Numbers in brackets indicate the number of subjects who took part



Figure - IIIg

Subject was fasted overnight and 10g of Lactulose (L), Raffinose (R) or Stachyose (S) in 150 ml of water was administered by mouth on different days. Breath samples for  $\text{H}_2$  measurement were taken at 10 minute intervals for about five hours.

CHAPTER IV

A D M I N I S T R A T I O N

O F

D I E T A R Y      F I B R E      I S O L A T E S

ACUTE ADMINISTRATION OF DIETARY FIBRE ISOLATES

Dietary fibre is a complex material made up of different polymers with varying molecular weight, chemical and physical properties (Cummings, 1976; Eastwood and Mitchell, 1976). The polymers have differing water holding capacity, adsorptive and gel forming properties, monofunctional or polyfunctional ion exchanger activity and a biological filter like function (Eastwood, 1973). Though these are in vitro findings, one might expect similar properties to manifest when dietary fibre passes down the gastrointestinal lumen. But, in the human intestine other factors also come into play. There will be interaction between each polymer, the polymers and intestinal bacteria, between polymer and solute, and the polymers, bacteria and solute as a whole (Eastwood, 1975). Consequently, the interaction of these chemicals along the gastrointestinal tract and the resulting effect on the physiological functions of the gastrointestinal system could be expected to be different. Also, the behaviour of these polymers in the intestine might vary when they are administered either separately, combined or as a complex dietary fibre. Lignin (Cymbaluk et al, 1973), silica (Van Soest and Jones, 1968) and cutin (Keys et al, 1970) have been found to affect the digestibility of other dietary fibre components. At present a satisfactory understanding of the different properties and the resulting effects in the gut of each individual component is lacking. Thus, it was thought worthwhile to administer well defined individual chemical components separately in a controlled acute situation and study their metabolism in the colon and its consequences. Different dietary fibre isolates were administered separately to fasted individuals and their colonic fermentation assessed indirectly by measuring breath  $H_2$  and  $CH_4$  excretion over a short period.

## MATERIALS AND METHODS

The participants in the study were 8 normal volunteers (4 males and 4 females) 23 to 47 years of age. They had no gastrointestinal problems and were in good health.

Ten or 20g of the fibre component were administered raw, mixed in 400 ml water with black currant syrup as flavouring. After an overnight fast, the test meals were taken at about 09.00 h in the morning and breath samples were obtained at hourly intervals thereafter for at least six hours. In some of the subjects prolonged sampling for up to twenty hours was performed. The dosage regimes were dictated by the difference in the gel forming properties of the different polymers, their availability and cost. The long fibered cellulose (Solka-Flok SW-40) and the substituted sodium carboxymethylcellulose, for example, have a high water holding capacity and gelling properties which make them difficult to take in 20g amounts. Pure hemicellulose is difficult to acquire in substantial amount and the pure oligosaccharides are very expensive. The flavouring was chosen by consensus as the preferred flavour. The time interval between each experiment was at least one week.

The control pattern of gas excretion was established with 400 ml water containing black currant syrup flavouring, the non-absorbable synthetic disaccharide lactulose and two types of potato based starch (Texrex Extender G and Celca-Sec - 500).

The substances studied were the oligosaccharide raffinose; the polysaccharides fibrous cellulose, microcrystalline  $\alpha$ -cellulose; sodium carboxymethylcellulose and high and low ester content pectin; and lignin (see Appendix 2 for detailed description of the chemicals).

Measurement used for comparison were the peak values for  $H_2$  and  $CH_4$ , which is the maximal level of the gases detected, and the total  $H_2$  and  $CH_4$  which is the summation of all values recorded during the test period.

## RESULTS

### Subjective Symptoms

Some subjects experienced abdominal distension and variable amounts of gaseousness, particularly with lactulose, raffinose and hemicellulose in that order. One had diarrhoea with lactulose.

### Breath Hydrogen

As shown in Table IVa, the control value for water with the flavouring alone was peak  $0.27 \pm 0.14$   $\mu\text{mol/l}$  ( $0.14 - 0.46$   $\mu\text{mol/l}$ ) and for the total  $1.00 \pm 0.73$   $\mu\text{mol/l}$  ( $0.23 - 1.91$   $\mu\text{mol/l}$ ). Starch did not significantly alter the excretion pattern, while lactulose considerably increased gas production, with breath  $H_2$  reaching values of  $4.90$   $\mu\text{mol/l}$  for the peak and  $16.36$   $\mu\text{mol/l}$  total.

The test substances cellulose (20g), pectin (20g) and lignin (20g) did not significantly alter the breath excretion pattern of  $H_2$ , while hemicellulose (10g) produced a peak excretion of  $0.77 \pm 0.59$   $\mu\text{mol/l}$  ( $0.14 - 1.36$   $\mu\text{mol/l}$ ) and a total of  $1.82 \pm 1.32$   $\mu\text{mol/l}$  ( $0.18 - 3.59$   $\mu\text{mol/l}$ ), and raffinose (10g) markedly increased breath  $H_2$  excretion to a peak of  $2.05 \pm 0.77$  ( $1.25 - 2.85$   $\mu\text{mol/l}$ ) and a total of  $5.64 \pm 2.09$   $\mu\text{mol/l}$  ( $3.21 - 8.32$   $\mu\text{mol/l}$ ). The different excretion patterns of the chemicals used is well illustrated by the histograms in Fig. IVa.

Prolonged follow up of some of the volunteers over 10 - 20 hours showed no delayed  $H_2$  increase for those substances which were not accompanied with increased gas production during the test period.

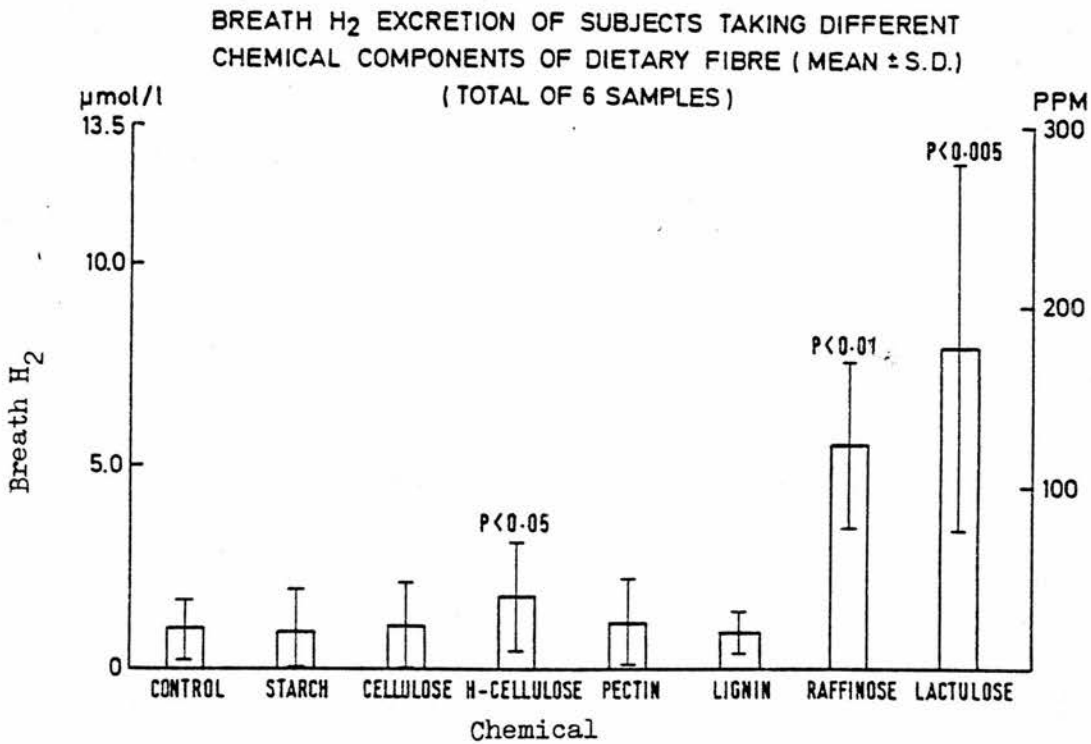
Table - IVa

Breath Hydrogen from subjects  
given different chemical components of dietary fibre  
(Mean  $\pm$  S.D.)

Chemical (20g)	No. of subjects	Peak Value ( $\mu\text{mol/l}$ )	Total Value (6 samples) ( $\mu\text{mol/l}$ )	
Control (water + flavouring)	8	$0.27 \pm 0.14$	$1.00 \pm 0.73$	
Starch	7	$0.27 \pm 0.32$	$0.86 \pm 1.14$	N.S.
Lactulose	6	$2.18 \pm 1.46$	$7.64 \pm 4.68$	$P < 0.005$
Raffinose*	6	$2.05 \pm 0.77$	$5.64 \pm 2.09$	$P < 0.01$
Cellulose	7	$0.36 \pm 0.23$	$1.09 \pm 1.09$	N.S.
Hemicellulose*	5	$0.77 \pm 0.59$	$1.82 \pm 1.32$	$P < 0.05$
Pectin	6	$0.36 \pm 0.23$	$1.18 \pm 1.09$	N.S.
Lignin	5	$0.36 \pm 0.18$	$0.91 \pm 0.59$	N.S.

\* 10g only in sample

Figure - IVa



10g or 20g of the test sample was ingested fasting (See Table IVa for details). The total for each individual was calculated from six hourly samples and the mean for the subjects involved calculated.

H - Cellulose = Hemicellulose.



### Breath Methane

Base line measurement of breath  $\text{CH}_4$  excretion showed that of the eight participants, three excreted more than  $0.90 \text{ umol/l}$ , two about  $0.50 \text{ umol/l}$  and three did not excrete any. Table IVb summarises the total  $\text{CH}_4$  excretion following the administration of the different chemicals for a representative of each of the three groups. There was no appreciable change in excretion for any of the substances. Figs. IVb and IVc contrast breath  $\text{H}_2$  and  $\text{CH}_4$  excretion in two subjects taking the series of carbohydrates and lignin. Though breath  $\text{H}_2$  excretion is markedly affected by some of the test substances,  $\text{CH}_4$  excretion remained the same, maintaining its individuality over a wide range of substances tested.

### Difference of Physical and Chemical Properties

The ingestion of polysaccharides of the same chemical group but with different physical properties, e.g. particle size and some chemical changes, e.g. ester content or substitution, did not make significant changes to the expired  $\text{H}_2$  or  $\text{CH}_4$  (Table IVc).



Table IVb

Total Breath Methano (umol/l) from subjects

Given different chemical components (20g) of dietary fibre

(6 samples)

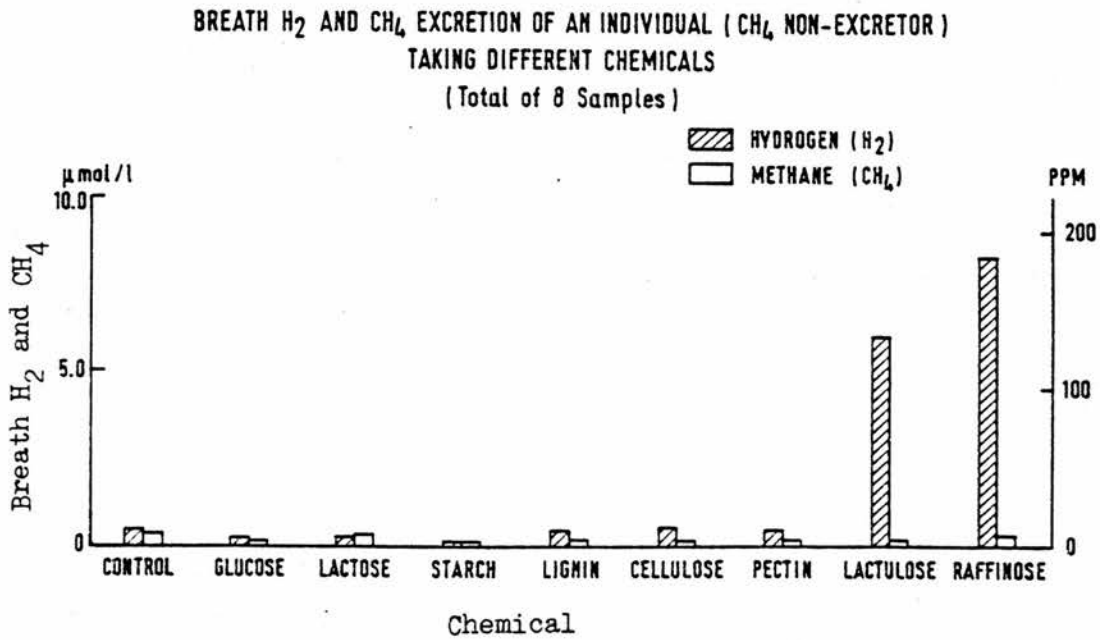
Subject	Control	Starch	Raffinose*	Lactulose	Cellulose	Hemi - Cellulose*	Pectin	Lignin
1	11.50	10.96	10.55	11.50	13.86	-	-	12.73
2	1.14	0.18	0.73	0	0.09	2.18	0.41	-
3	0	0.09	0.23	0	0.09	0.50	-	0

\* 10g only administered

The three subjects are representative of each group of CH<sub>4</sub> excretors.

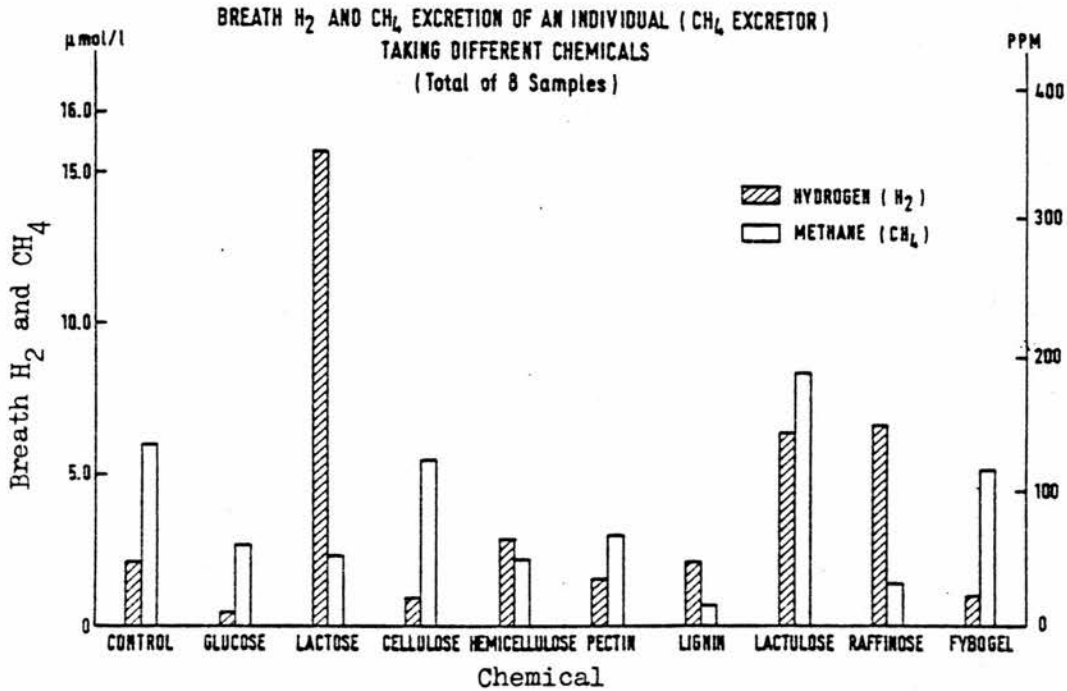
Values are total of six samples taken at hourly intervals.

Figure - IVb



The test substance was administered to the same fasting individual on different days. Control was 300 ml water with flavouring. Lactulose and raffinose were given 10g each; starch, lignin, cellulose and pectin - 20g each; glucose and lactose - 50g each. Breath samples were taken at hourly intervals for eight hours.

Figure - IVc



The test substance was administered to the same fasting individual on different days. Control was 300 ml water with flavouring. Lactulose, raffinose and hemicellulose were given 10g each; cellulose, pectin and lignin - 20g each; glucose and lactose - 50g each; fybogel (Ispaghula husk) - 7g. Breath samples were taken at hourly intervals for eight hours. Note subject is lactase deficient.

Table - IVc

Breath Hydrogen From Subjects Given  
Different Types of a Dietary Fibre Isolate  
(Mean  $\pm$  S.D.)

Chemical	Type* (20g)	No. of subjects	Peak Value ( $\mu\text{mol/l}$ )	Total Value (6 samples) ( $\mu\text{mol/l}$ )
Starch	fine C-S 500	4	$0.32 \pm 0.41$	$0.90 \pm 1.26$
	Coarse T-E G	3	$0.23 \pm 0.27$	$0.77 \pm 1.17$
Cellulose	AV - 101	3	$0.36 \pm 0.27$	$1.04 \pm 0.72$
	AV - 102	3	$1.41 \pm 0.23$	$1.44 \pm 1.53$
	SW - 40**	3	$0.63 \pm 0.45$	$1.26 \pm 0.45$
	Na COOH-ME**	4	$0.45 \pm 0.45$	$0.90 \pm 1.17$
Pectin	low ester L.M.-3466	2	$0.54 \pm 0.45$	$1.76 \pm 0.96$
	high ester N.F.-3442	4	$0.27 \pm 0.05$	$0.54 \pm 0.32$

\* See appendix for details

\*\* 110g only administered

CHAPTER V

ADMINISTRATION  
OF  
WHOLE FIBRE

ADMINISTRATION OF WHOLE FIBRE

The bacterial metabolism of dietary fibre and its effects on the physiological functions of the gastrointestinal system, when given whole and in its natural form may be quite different from that of extracts or synthesised fibre components. Sources of dietary fibre are varied and the proportions of the constituent polymers vary too. The maturity of the plant and even the part from where the sample is taken determines the chemical composition, the amount and the proportion of the fibre components (Cummings, 1976). These differences must have a bearing on the physico-chemical properties of the dietary fibre source and indeed there is ample evidence for this in vitro studies (Eastwood and Mitchell, 1976).

Also most of what we know about dietary fibre metabolism and its effects on the physiological functions of the colon in man are either extrapolations from ruminant physiology or derived from findings of what goes out in the faeces and to a lesser extent from investigations on the accessible parts of the colon, i.e. the left side. However, most of the bacterial activity and absorption of water, electrolytes and the remaining bile acids occur in the right side (particularly the caecum) rather than the left side of the colon. It appears therefore that dietary fibre breakdown and its effect on colon function is best studied in this part. But in man, this part of the colon is not easily accessible and either an indirect method of observation or an animal model is required. In this project these two approaches were employed to study different aspects of bacterial breakdown of dietary fibre and its consequences on colon function.

In experiments in man, caecal fermentation of dietary fibre was gauged indirectly by measuring breath  $H_2$  and  $CH_4$  and changes in

colon function by measuring faecal bile acid excretion pattern, intestinal transit time, stool weight and other variables in the faeces. While in animal experiments the effect of dietary fibre on bile acid metabolism alone and the role of the colon in this process was investigated in detail. Since testing all dietary fibre sources is beyond the scope of the project, two sources of dietary fibre - carrot and wheat bran - were chosen. The two sources were selected because their physical and chemical properties have been comparatively well documented in our laboratory and elsewhere. As the effect of bran in man is relatively well known and since preliminary testing showed no effect on the excretion pattern of the two gases, it was used in experiments on the rat. The carrot was administered to human volunteers.

## A. HUMAN EXPERIMENTS

The effect of acute and chronic administration of carrot was studied on the pattern of breath  $H_2$  and  $CH_4$  excretion. In the chronic administration experiment variables other than the two gas measurements were looked into viz. serum cholesterol, triglycerides, phospholipids and carotene, wet and dry stool weight, faecal bile acids, fat and electrolytes, and intestinal transit time. The effect of cooking the carrot on breath  $H_2$  and  $CH_4$  excretion was also studied in acute experiments.

### MATERIALS AND METHODS

The carrot used was Flako Autumn King, cropped at 200 days and the details of its chemical and physical properties are given in Appendix 3.

Eight subjects were studied whilst carrying out their normal routine in the hospital service. They were aged between 25 to 41 years and all within 14% of their ideal body weight.

In the acute experiment, breath samples were taken at hourly intervals over 8 hours on three separate days, with at least one week interval between each study. The first day was the control day when breath  $H_2$  and  $CH_4$  were measured without eating carrot but otherwise taking their usual meals. On the second study day 200g raw carrot was eaten as a supplement to their usual meal and breath gases measured. On the third study day 200g boiled carrot was administered in the same manner and gas tests performed as above.

The experimental design for the chronic experiment consisted of one week control period followed by three weeks with supplement of 200g raw carrot each day, eaten at breakfast time. During the study period breath samples were analysed hourly for 8 hours on two separate days of each week. The gas measurements for each week



are the mean of two days measurement.

Blood samples were collected on three occasions after 15 hours fasting. One was taken in the period of control, the second in the third week on carrot and the last three weeks after stopping the carrot. The serum was used for measurement of cholesterol (Roschlau et al, 1974), triglycerides (Eg<sup>g</sup>stein, 1966), phospholipids (Zilversmit and Davis, 1950), and carotene (Varley, 1967) in the Chemistry Laboratory of the Western General Hospital.

All stool excreted over a seven day period were collected during the control period, during the third week of the carrot administration and the third week after stopping carrot. The pooled faecal sample was frozen at  $-20^{\circ}\text{C}$  and freeze-dried before analysis of bile acids (Evrard and Janssen, 1968), fat (Varley, 1967), and electrolytes by flame photometry and atomic absorption spectrophotometry after charring with nitric acid. At the same time whole gut transit time were measured using barium impregnated markers (Hinton et al, 1969). Forty cubical pellets (sp.gr. 1.1) were administered on the first day of collection and time noted. The number of pellets passed in the stool were counted whilst made visible by fluoroscopy. The time when 80% of the pellets were passed was taken as the transit time. These markers also gave an indication of the completion of collection.

Dietary histories were taken both by one week retrospective diet history gathered by a dietician and by a diet diary kept over one week. The daily dietary intake was calculated from the food tables of McCance and Widdowson (1960) and the guide to calculating intakes of dietary fibre by Southgate et al (1976<sup>a</sup>).

The results are given as the mean and standard error of the mean and the significance estimated by Wilcoxon's matched pairs

signed rank test.

## RESULTS

### 1. Acute Study

Table Va, shows the breath  $H_2$  excretion values for the five subjects. Though two of the participants (A & D) showed marked increase, no overall statistical significant change could be detected in the level of  $H_2$  excretion on taking 200g supplementary carrot raw or cooked.  $CH_4$  excretion was unaffected.

### 2. Chronic Study

The results for the dietary history are given in Table Vb. It shows that it is typical for urban middle class population and the range of values for the individual members is as could be expected. As far as ascertained, the subjects remained on the same type of dietary habit throughout the period of study and there was no significant change in any of the dietary constituents assessed.

Table Vc shows the  $H_2$  changes for the five subjects studied. Taking 200g raw carrot daily for three weeks had some effect on the excretion of breath  $H_2$ . The total breath  $H_2$  did not increase markedly over the base line value during the first week of carrot administration but increased significantly during the second and third week. Breath  $CH_4$  excretion was unaffected and remained individual (Table Vd). The mean values of  $H_2$  and  $CH_4$  excretion for the five subjects are plotted as histograms in Fig. Va and illustrate well the difference in the excretion pattern of the two gases.

Total faecal bile acid excretion increased with the intake of carrot in four of the five subjects studied and had not returned to base line values after three weeks without supplementary carrot

Table - Va

Total Breath H<sub>2</sub> Excretion (umol/l)  
After Acute Administration of 200g Carrot  
 (8 samples)

Subject	Control Day	Raw Carrot	Cooked Carrot
A	0.05	0.72	0.36
B	1.17	0.54	0.99
C	4.28	3.02	3.02
D	3.83	7.83	5.27
E	0.23	0.45	0.59
Mean	1.91	2.51	2.05
S.D.	2.01	3.16	2.08
S.E.M.	0.90	1.41	0.93

Table - Vb

Dietary Intake per 24 hours of the 5 Subjects  
Involved in the Chronic Carrot Experiment

Item	Mean	Range
Protein (g)	81	64 - 103
Fat (g)	104	76 - 154
Saturated	95	66 - 148
Unsaturated	9	6 - 12
Carbohydrate (g)	260	203 - 320
Energy (kJ)	10,000	8,570 - 10,900
Cholesterol (mg)	534	371 - 700
Dietary Fibre (g)	21	14 - 33
Non-cellulosic	15	9 - 22
Cellulose	5	3 - 8
Lignin	2	1 - 3
Hexoses	7	5 - 9
Pentoses	5	3 - 7
Phytate (mmol)	0.26	0.09 - 0.40
Calcium (mmol)	24.4	18.0 - 33.3
Copper (mmol)	0.025	0.018 - 0.034

Values were estimated from retrospective diet history plus a diet diary kept over one week.

Table - Vc

Total Breath H<sub>2</sub> Excretion (umol/l)  
by Subjects Taking 200g Raw Carrot Daily  
 (8 samples)

Subject	Control Week	First Week	Second Week	Third Week
A	0	0.14	2.90	0.23
B	0.27	1.96	2.30	3.94
C	2.63	2.77	3.00	3.44
D	0.47	0.38	2.43	0.68
E	0.45	0	0.23	0.59
Mean	0.77	1.05	2.17	1.77
S.D.	1.06	1.24	1.13	1.77
S.E.M.	0.47	0.55	0.55	0.79

Values for each week are the average of two days test results.

Breath samples were taken at hourly intervals for eight hours.

Table - Vd

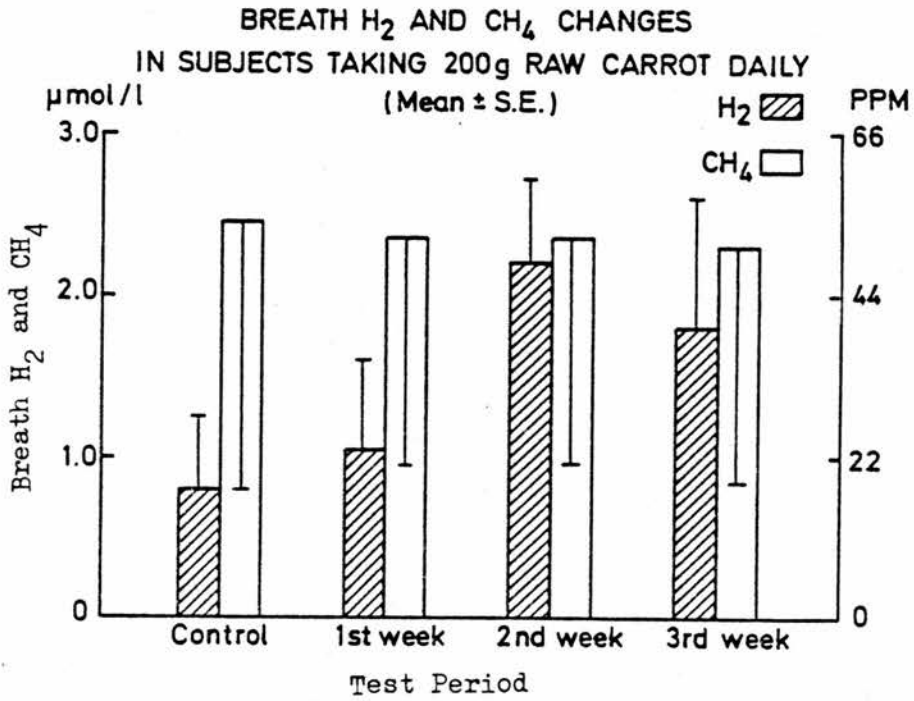
Total Breath CH<sub>4</sub> Excretion (umol/l)  
by Subjects Taking 200g Raw Carrot Daily  
 (8 samples)

Subject	Control Week	First Week	Second Week	Third Week
A	8.41	7.57	7.48	7.79
B	0	0	0	0
C	3.20	2.98	2.74	2.05
D	0.73	1.17	1.47	1.60
E	0	0	0	0
Mean	2.47	2.34	2.34	2.30
S.D.	3.57	3.17	3.09	3.21
S.E.M.	1.60	1.41	1.38	1.43

Values for each week are the average of two days test results.

Breath samples were taken at hourly intervals for eight hours.

Figure - Va



Values for each week are the average of two days results. Breath samples were taken hourly for eight hours and the mean is calculated on the total eight samples.



(Table Ve). The same applies to each individual bile acids. There is proportionally more increase in the primary bile acids excretion as compared to the secondary bile acids (85% and 30% respectively).

The stool wet weight significantly increased with carrot intake and decreased again three weeks after cessation of carrot (Table Vf). However, the small increase in stool dry weight was not statistically significant. There was no effect on the whole gut transit time. Recovery of markers was on the average about 88%. Faecal fat excretion increased from a mean of 5.14  $\mu\text{mol}/24\text{h}$  during the control period to 7.22  $\text{mmol}/24\text{h}$  with the carrot intake and remained high three weeks after (6.0  $\text{mmol}/24\text{h}$ ), though this was not significantly higher than the base line value.

The serum cholesterol concentration decreased slightly with carrot intake and remained so three weeks after stoppage of carrot. There was no effect on the triglycerides and phospholipids (Table Vg). The mean serum carotene increased from  $2.5 \pm 0.4 \mu\text{mol}/\text{l}$  to  $3.8 \pm 0.8 \mu\text{mol}/\text{l}$  with carrot intake. The increase is well below the level considered toxic (greater than 10  $\mu\text{mol}/\text{l}$ ).



Table - Ve

Faecal Bile Acid Excretion Pattern (mmol/24hours)  
of 5 Subjects Taking 200g Raw Carrot Daily  
 (Mean + S.E.M.)

Bile Acid	Control	Carrot	Control
Cholic	0.02 $\pm$ 0.01	0.05 $\pm$ 0.01	0.05 $\pm$ 0.05
Chenodeoxycholic	0.05 $\pm$ 0.05	0.08 $\pm$ 0.02	0.07 $\pm$ 0.02
Lithocholic	0.24 $\pm$ 0.01	0.30 $\pm$ 0.04	0.28 $\pm$ 0.02
Deoxycholic	0.34 $\pm$ 0.05	0.48 $\pm$ 0.07	0.43 $\pm$ 0.02
Total	0.66 $\pm$ 0.07	0.93 $\pm$ 0.08	0.82 $\pm$ 0.03

Samples were analysed from seven days stool collections  
 (the last week for the carrot ingestion).

Table - VfStool Changes of 5 SubjectsTaking 200g Supplementary Carrot Daily(Mean  $\pm$  S.E.M.)

	Control	Carrot	Control
Wet weight (g)	142 $\pm$ 37	177 $\pm$ 33*	145 $\pm$ 15
Dry weight (g)	35 $\pm$ 6	41 $\pm$ 6	39 $\pm$ 2
Fat (mmol)	5.14 $\pm$ 1.04	7.22 $\pm$ 1.6*	6.00 $\pm$ 1.20
Neutral steroids (mmol)	2.20 $\pm$ 0.21	2.46 $\pm$ 0.28	2.58 $\pm$ 0.37
Transit time (days)	3.0 $\pm$ 0.5	2.3 $\pm$ 0.6	2.3 $\pm$ 0.3

\*p &lt; 0.05

The different values were calculated from 7 days collection of stool of each individual and then the mean calculated for the group.

Table - Vg

Changes in Serum Lipids (mmol/l) and Carotene (umol/l)  
of 5 Subjects Taking 200g Raw Carrot Daily  
 (Mean  $\pm$  S.E.M.)

	Control	Carrot	Control
Cholesterol	6.6 $\pm$ 0.5	5.9 $\pm$ 0.3*	6.0 $\pm$ 0.3
Triglyceride	1.3 $\pm$ 0.3	1.4 $\pm$ 0.4	1.2 $\pm$ 0.3
Phospholipid	3.2 $\pm$ 0.1	3.1 $\pm$ 0.1	3.1 $\pm$ 0.3
Carotene	2.5 $\pm$ 0.4	3.8 $\pm$ 0.8*	2.5 $\pm$ 0.3

\*p 0.05

Blood samples were taken one day during the study period  
 (the third week during carrot intake) after overnight fasting.

## B. RAT EXPERIMENTS

In vitro studies on the inter-relationship between dietary fibre and bile acids, have shown that fibre adsorbs bile acids (Kritchevsky and Story, 1974). The adsorption is greater for unconjugated bile acids, particularly deoxycholic acid and is pH dependent (Eastwood and Mitchell, 1976). Lignin binds bile acids more than other polymers of dietary fibre (Eastwood and Hamilton, 1968). Studies in vivo both in man and the rat indicate that the quantity of fibre in the diet can influence the rate of synthesis and half life of bile acids (Danielsson, 1973b). The addition of bran to a semi-synthetic diet given to rats can result in an increase of the total bile acids in the small intestine (Eastwood and Boyd, 1967). A pellet diet increased the excretion of labelled cholic acid and metabolites when compared with fibre depleted semi-synthetic diet, while addition of 20% cellulose to the semi-synthetic diet did not significantly affect the excretion level (Gustafsson and Norman, 1969).

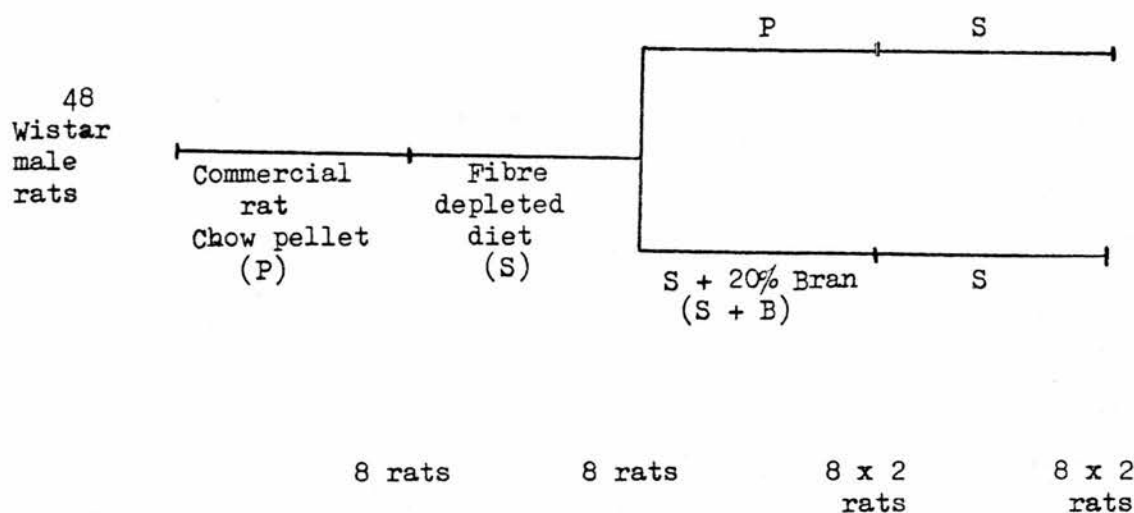
In the rat information has been gathered on the effect of dietary fibre on bile acid metabolism in the small intestine and its excretion in the faeces. However, the role of the colon and the mechanism by which fibre affects bile acid synthesis are not clear. This may in part be due to the available methods of bile acid analysis, which until recently, were unable to differentiate all the bile acid compounds found in the rat intestine, particularly the muricholic group of bile acids (Madsen et al, 1976).

In this study an attempt was made to look at the role of the colon in bile acid metabolism by measuring bile acids in the small intestine and colon separately. Also, the effect of bran on the

rate limiting enzyme of bile acid synthesis, liver cholesterol 7 $\alpha$ -hydroxylase, was determined.

#### METHOD

Forty eight mature male rat of Wistar strain were used. They were kept in a constantly lighted animal house and feeding and water was unrestricted. At the start of the experiment the rats weighed about 200g and received the different dietary regimes in the following order:-



The major constituents of the different diets are given in Table Vh.

At the end of each period of two weeks, eight rats were killed with ether, weighed and the liver and the intestine removed. The liver was immediately chilled by placing in ice-cold 0.25M sucrose. A one gram segment was then cut and homogenised in sucrose solution (20% w/v). The microsomal fraction of the homogenate was separated by repeated ultra-centrifugation and then mixed with 4-<sup>14</sup>C cholesterol. The mixture was incubated in air and at 37°C for 60 minutes. The reaction was stopped by adding methanol. After extracting the lipid fraction, sterols were separated on thin layer

Table - Vh

Nutritional Composition of <sup>the</sup> Different  
Dietary Regimes  
 (g/100g edible substance)

Nutrient	Commercial Chow Pellet (P)	Fibre Depleted Diet (S)	S + 20% Bran (S+B)
Carbohydrate	60.0	64.6	61.0
Protein	19.0	20.2	19.1
Fat	2.0	1.8	2.4
Acid detergent fibre	5.9	2.1	4.1
Lignin	1.2	0.4	1.0
Moisture	10.0	9.5	9.7
Others	3.0	1.8	3.7

chromatograph (TLC). The cholesterol, 7 $\alpha$ -hydroxycholesterol and a "Blank" fraction were scraped separately into liquid scintillation vials and radio-activity counted. The cholesterol 7 $\alpha$ -hydroxylase activity was expressed as a percentage conversion of 4-<sup>14</sup>C cholesterol to 7 $\alpha$ -hydroxy (4-<sup>14</sup>C) - cholesterol (Mitton et al, 1971).

The small intestinal and large intestinal contents were washed out with distilled water into separate containers, freeze-dried and the bile acid content analysed as described in the methods section.

The bile acid values for the individual rats with each dietary regime were pooled, separately for the small intestine and colon. The total bile acid per rat, the total bile acid per gram body weight, the total bile acid pool (i.e. small intestine + colon) per rat, the percentage of each individual bile acid and the proportion of the primary, secondary and muricholic group of bile acids were calculated for each group and compared. With respect to the liver cholesterol 7 $\alpha$ -hydroxylase enzyme, the mean level of activity for each group of rats was calculated and comparison made between the groups.

The statistical analyses used were the mean and standard error of the mean, and significance was estimated by the student "t" test.

## RESULTS

### 1. General

All the rats appeared to be fit on the different dietary regimes. Their body weight increased at a constant rate during the period of study and at the last killings the rats have achieved an average weight of about 350g. The total dry weight of the intestinal contents was greater on high fibre diet than on a fibre depleted diet. This was more reflected in the small intestinal content than the colonic (Tables Vi, Vj).

Table - Vi

Composition of Bile Acids in Small Intestine  
(Mean  $\pm$  S.E.)

Bile Acids	Diet Sequence					
	Commercial Rat Pellet Days 1-14	Fibre Depleted Diet Days 15 - 28	F.D.D. + 20% Bran Days 29 - 42	Fibre Depleted Diet Days 43 - 56	Commercial Rat Pellet Days 29-42	Fibre Depleted Diet Days 43 - 56
Total (umols/rat) Total (umols/100g body weight)	39.9 $\pm$ 3.5 19.7 $\pm$ 1.3	18.8 $\pm$ 2.8 7.2 $\pm$ 0.9	45.0 $\pm$ 3.4 14.7 $\pm$ 0.8	28.7 $\pm$ 3.5 8.2 $\pm$ 0.8	51.3 $\pm$ 4.9 17.9 $\pm$ 2.4	29.5 $\pm$ 2.7 8.8 $\pm$ 0.8
% cholic	48.4 $\pm$ 1.2	45.2 $\pm$ 3.7	48.9 $\pm$ 1.8	44.4 $\pm$ 2.4	46.4 $\pm$ 1.0	45.1 $\pm$ 2.6
% chenodeoxycholic	9.7 $\pm$ 1.0	7.5 $\pm$ 1.1	7.5 $\pm$ 0.5	5.1 $\pm$ 0.5	9.5 $\pm$ 1.0	4.3 $\pm$ 0.7
% deoxycholic	1.5 $\pm$ 0.1	1.4 $\pm$ 0.1	1.1 $\pm$ 0.2	1.4 $\pm$ 0.2	1.8 $\pm$ 0.1	2.6 $\pm$ 0.5
% hydoxycholic	5.9 $\pm$ 0.6	0.5 $\pm$ 0.3	7.7 $\pm$ 1.2	3.0 $\pm$ 0.7	6.7 $\pm$ 0.5	2.0 $\pm$ 0.2
% $\alpha$ -muricholic	11.7 $\pm$ 1.0	16.5 $\pm$ 1.6	11.3 $\pm$ 0.5	12.9 $\pm$ 1.2	11.5 $\pm$ 1.2	12.5 $\pm$ 1.4
% $\beta$ -muricholic	20.6 $\pm$ 1.5	21.2 $\pm$ 1.3	19.3 $\pm$ 1.7	22.2 $\pm$ 1.2	21.0 $\pm$ 1.6	23.0 $\pm$ 1.1
% $\omega$ -muricholic	2.3 $\pm$ 0.4	7.9 $\pm$ 0.8	3.2 $\pm$ 0.4	11.1 $\pm$ 1.2	3.1 $\pm$ 0.7	10.4 $\pm$ 0.9
Wt. of dry contents (g)	0.78 $\pm$ 0.05	0.18 $\pm$ 0.03	0.57 $\pm$ 0.04	0.50 $\pm$ 0.05	1.14 $\pm$ 0.13	0.45 $\pm$ 0.03

8 rats were involved in each group



Table - VI

## Composition of Bile Acids in Colon

(Mean  $\pm$  S.E.)

Bile Acids	Diet Sequence					
	Commercial Rat Pellet Days 1-14	Fibre Depleted Diet Days 15 - 28	F.D.D. + 20% Bran Days 29 - 42	Fibre Depleted Diet Days 43 - 56	Commercial Rat Pellet Days 29-42	Fibre Depleted Diet Days 43 - 56
Total (umols/rat) Total (umols/100g body weight)	7.8 $\pm$ 1.5 3.8 $\pm$ 0.7	39.8 $\pm$ 6.4 15.2 $\pm$ 2.2	13.1 $\pm$ 3.1 4.2 $\pm$ 0.9	24.0 $\pm$ 2.8 7.0 $\pm$ 0.7	14.2 $\pm$ 2.8 4.3 $\pm$ 0.8	30.7 $\pm$ 4.2 9.5 $\pm$ 1.6
% cholic	7.9 $\pm$ 0.7	16.3 $\pm$ 1.9	10.7 $\pm$ 1.6	6.2 $\pm$ 1.4	13.8 $\pm$ 2.8	5.1 $\pm$ 0.9
% chenodeoxycholic	1.3 $\pm$ 0.4	2.4 $\pm$ 0.3	2.7 $\pm$ 0.6	1.6 $\pm$ 0.6	2.7 $\pm$ 0.7	0.9 $\pm$ 0.1
% deoxycholic	21.7 $\pm$ 0.8	3.5 $\pm$ 0.5	9.5 $\pm$ 1.4	10.2 $\pm$ 1.0	18.7 $\pm$ 1.8	10.7 $\pm$ 1.1
% lithocholic	11.6 $\pm$ 1.3	1.1 $\pm$ 0.2	3.6 $\pm$ 0.9	4.1 $\pm$ 1.4	10.5 $\pm$ 1.7	2.0 $\pm$ 0.3
% hydoxycholic	26.8 $\pm$ 1.8	2.5 $\pm$ 0.2	13.7 $\pm$ 3.1	8.4 $\pm$ 3.2	25.9 $\pm$ 1.3	3.2 $\pm$ 0.8
% a-muricholic	2.7 $\pm$ 0.6	11.4 $\pm$ 0.7	5.2 $\pm$ 1.1	4.7 $\pm$ 0.5	1.9 $\pm$ 0.5	6.2 $\pm$ 0.6
% $\beta$ -muricholic	10.4 $\pm$ 1.7	27.3 $\pm$ 2.4	18.4 $\pm$ 3.3	14.0 $\pm$ 2.6	9.2 $\pm$ 1.2	27.1 $\pm$ 3.3
% $\mu$ -muricholic	19.6 $\pm$ 3.3	35.5 $\pm$ 3.6	36.2 $\pm$ 2.6	50.7 $\pm$ 4.8	17.1 $\pm$ 3.2	45.3 $\pm$ 3.0
Wt. of dry contents (g)	0.98 $\pm$ 0.09	1.28 $\pm$ 0.11	1.52 $\pm$ 0.18	1.65 $\pm$ 0.14	1.60 $\pm$ 0.26	1.57 $\pm$ 0.14

8 rats were involved in each group

## 2. Total Intestinal Bile Acids

Tables Vi and Vj summarise the results of the bile acid analysis on the small intestinal and colonic contents. When the rats were fed on the chow pellet (P) diet, there were more bile acids in the small intestine compared with the colon, while on the fibre depleted (S) diet there were more bile acids in the colon. This was true both for the total bile acids per rat or per gram body weight (Figs. Vb and Vc). Supplementing the fibre depleted diet with 20% bran (S + B) resulted in bile acid values similar to that of the chow (P) diet. The bile acid pool did not significantly alter on changing from one dietary regime to another.

## 3. Individual Bile Acids

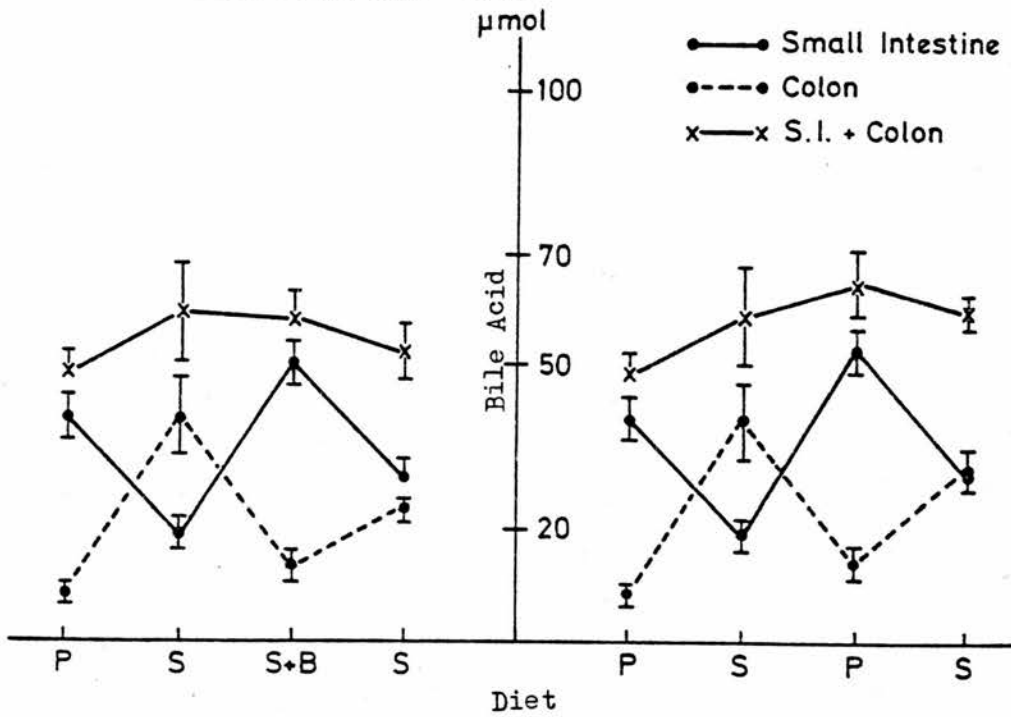
The relative proportion of hyodeoxycholic acid in the small intestine was greater on chow ( $5.9 \pm 0.6\%$ ) than on the soft diet ( $0.5 \pm 0.3\%$ ) while the reverse applies to  $\omega$ -muricholic acid ( $P = 2.3 \pm 0.4\%$ ;  $S = 7.9 \pm 0.8\%$ ) and to lesser extent to  $\alpha$ -muricholic acid ( $P = 11.7 \pm 1.0\%$ ;  $S = 16.5 \pm 1.6\%$ ). The other bile acids in the small intestine did not show remarkable changes (Table Vi).

In the colon the relative proportions of cholic, chenodeoxycholic and all the muricholic acids were generally low on chow and high on soft diet. On the other hand, the relative amounts of lithocholic, deoxycholic and hyodeoxycholic acids were higher on chow pellet than on the fibre depleted diet (Table Vj).

Two weeks on bran supplemented soft diet (S + B) was followed by a pattern of bile acid distribution in the small intestine similar to that of the chow diet (Table Vi). But, in the colon though the general picture was the same as the chow pellet, the degree of change was markedly different (Table Vj). Also, the  $\omega$ -muricholic level was more comparable to that of the soft diet regime than the

Figure - Vb

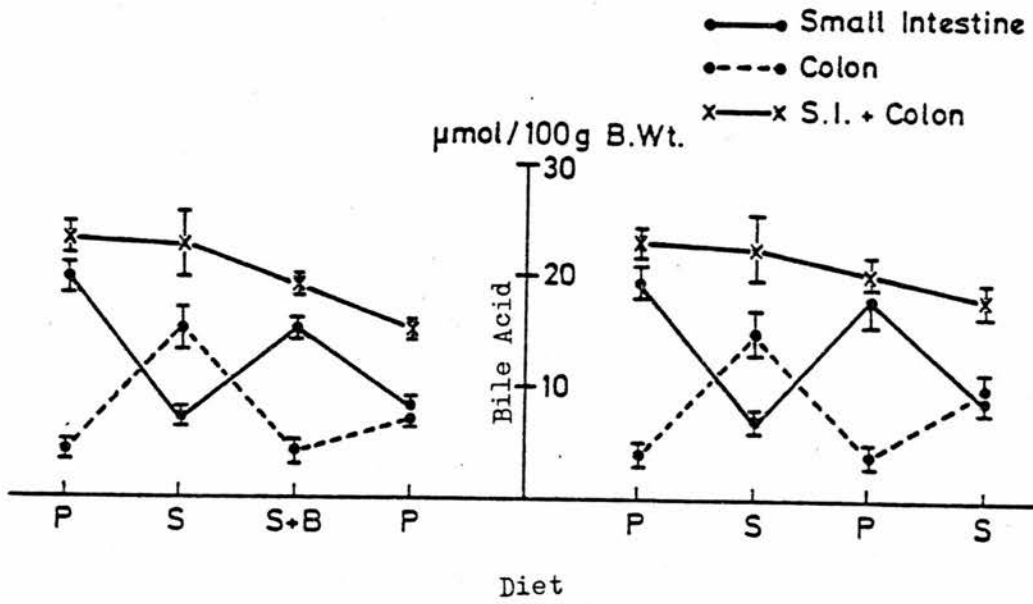
TOTAL BILE ACID CONTENTS OF RATS (n=8) ON DIFFERENT DIETARY REGIMES (Mean  $\pm$  S.E.)



8 rats were killed at the end of each two weeks dietary regime - commercial chow pellet (P), fibre depleted diet (S), S + 20% bran (S+B). See Table Vh for details of dietary constituents.

Figure - Vc

TOTAL BILE ACID CONTENTS OF RATS (n=8) ON DIFFERENT  
DIETARY REGIMES (Mean  $\pm$  S.E.)



8 rats were killed at the end of each two weeks dietary regime - commercial chow pellet (P), fibre depleted diet (S), S + 20% wheat bran (S + B). See Table Vh for details of dietary constituents.

chow ( $S = 35.5 \pm 3.6\%$ ;  $S + B = 36.2 \pm 2.6\%$ ;  $P = 19.6 \pm 3.3\%$ ).

On changing from the bran ( $S + B$ ) regime to fibre depleted regime, relative changes of the individual bile acids were less marked except for  $\mu$ -muricholic acid which increased significantly (Tables Vi and Vj).

#### 4. Bile Acid Groups

When the bile acid values for each group of rats was divided into primary, secondary and muricholic groups of bile acids, the picture was as follows:

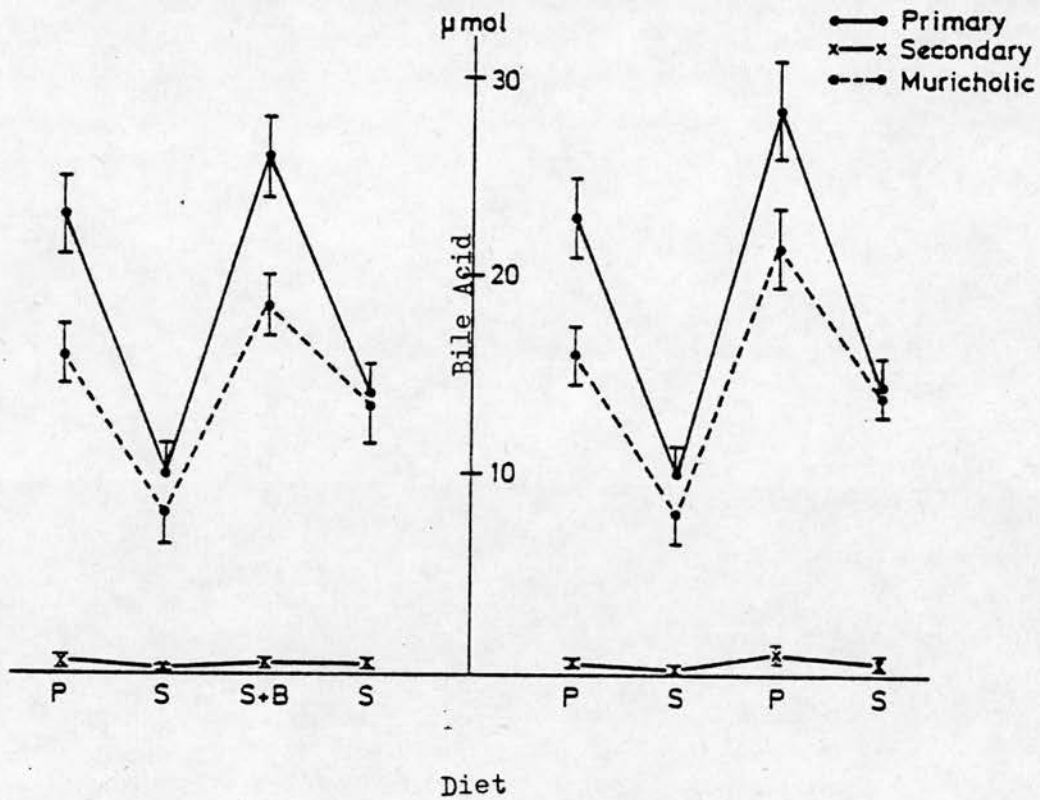
- (a) In the small intestine, all three bile acid groups were greater on chow pellet or the bran supplemented soft diet than on the soft diet alone (Fig. Vd). Conversely in the colon, the level of primary and the muricholic group of bile acids were smaller on chow or bran diets than on soft diet, though the secondary bile acids remained greater on the high fibre diets ( $P$  or  $S + B$ ) than on the fibre depleted diet ( $S$ ) (Fig. Ve).
- (b) Adding the small intestinal and the colon values for each group of bile acids showed that the primary and secondary bile acids were raised on chow or bran supplemented diets, whilst the muricholic group of bile acids was increased on fibre depleted diet as compared to high fibre diet (Fig. Vf).
- (c) The proportional amounts of the muricholic group of bile acids were very high on all dietary regimes, amounting to 30-50%.

#### 5. Liver Cholesterol 7 $\alpha$ -Hydroxylase Activity

The activity of this enzyme, as expressed in percentage conversion, was significantly higher when the rats were fed on the chow pellet ( $4.92 \pm 0.47\%$ ) than on the fibre depleted diet ( $2.71 \pm 1.20\%$ ). There was no statistically significant increase in the level of activity on switching the rats from soft to 20% bran supplemented

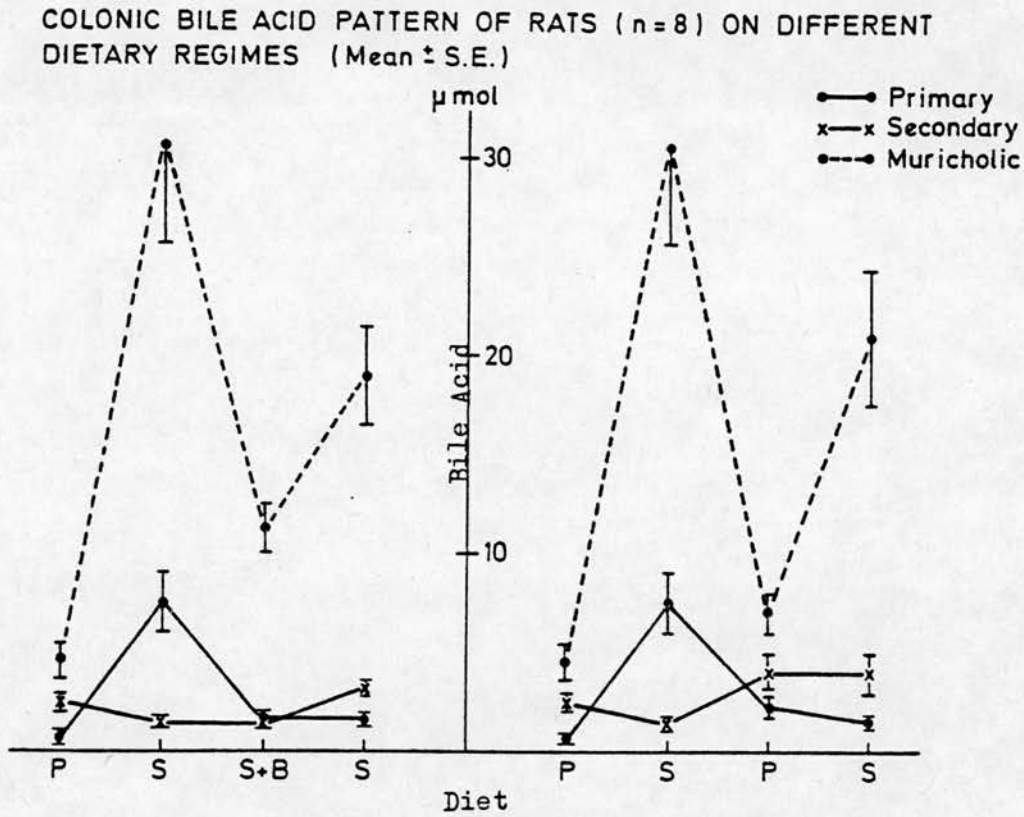
Figure - Vd

SMALL INTESTINAL BILE ACID PATTERN OF RATS (n=8) ON  
DIFFERENT DIETARY REGIMES (Mean  $\pm$  S.E.)



8 rats were killed at the end of two weeks on different diets - commercial chow pellet (P), fibre depleted diet (S), S + 20% wheat bran (S + B). See Table Vh for details of dietary constituents. Primary bile acids (C & CDC), Secondary (DC) and muricholics ( $\alpha$ ,  $\beta$ , W-MC and HDC).

Figure - Ve

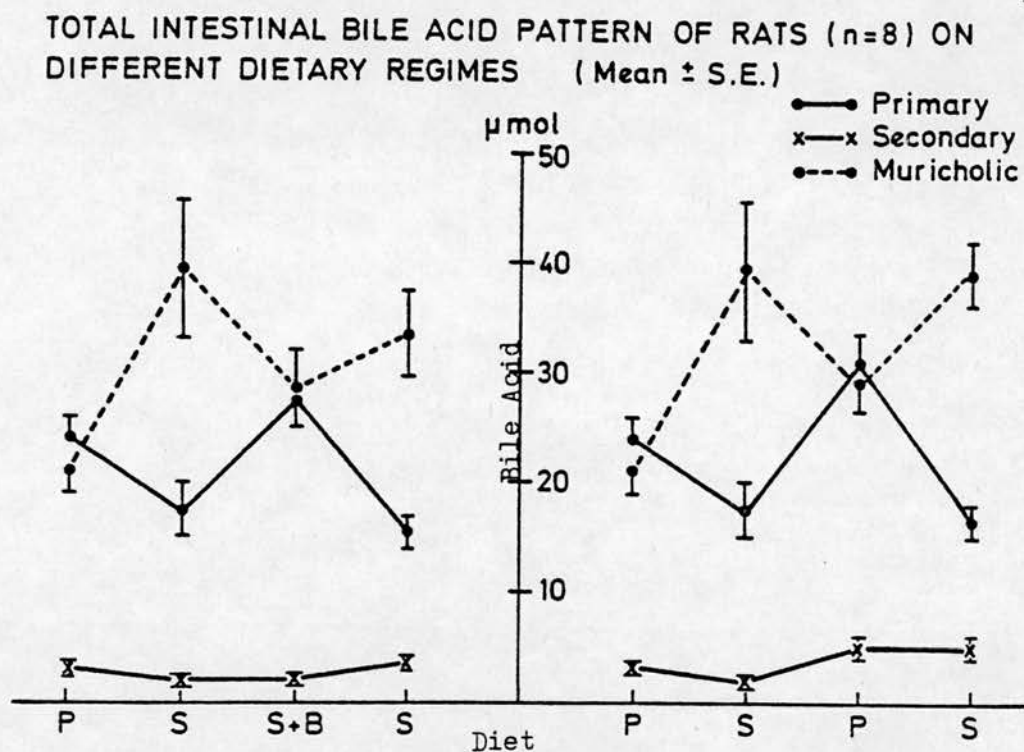


8 rats were killed at the end of two weeks on different diets - commercial chow pellet (P), fibre depleted diet (S), S + 20% wheat bran (S + B). See Table Vh for details of constituents.

Primary bile acids (C & CDC), secondary (DC & LC) and muricholics ( $\alpha$ -,  $\beta$ -,  $\omega$ -MC and HDC).



Figure - Vf

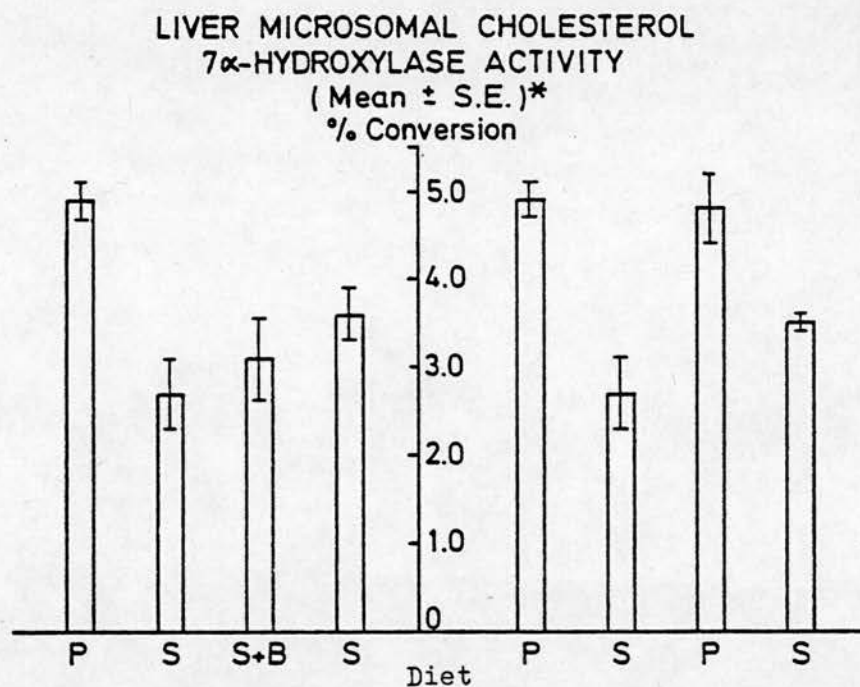


8 rats were killed at the end of two weeks on different diets - commercial chow pellet (P), fibre depleted diet (S), S + 20% wheat bran (S + B). See details of constituents in Table Vh.

Primary bile acids (C & CDC), secondary (DC & LC) and muricholics ( $\alpha$ -,  $\beta$ -,  $\omega$ -MC and HDC).



soft diet ( $3.08 \pm 0.90\%$ ). The relative activity, in fact, appeared to have increased when the rats were put back on the soft diet after having been on the bran supplemented soft diet for two weeks (Fig. Vg).

Figure - Vg

8 rats were killed at the end of two weeks on each diet - commercial chow pellet (P), fibre depleted diet (S), S + 20% wheat bran (S + B). Enzyme activity was measured for each rat and the mean value calculated for the group. Number of samples for the first P and S diets were 8, for S + B and the second P and S diets 4.

\* % conversion of 4-<sup>14</sup>Ccholesterol to 7 $\alpha$ -hydroxy(4-<sup>14</sup>C)-cholesterol.

CHAPTER VI

DISCUSSION

## DISCUSSION

### General

The effect of dietary fibre on colon function, particularly as reflected on stool weight, has been appreciated since antiquity (McCance and Widdowson, 1956), but the mechanism involved in the process has up to the present been speculative. This is because of the lack of sufficient knowledge about the chemical nature of fibre, the metabolic changes it undergoes in the colon and even about the normal physiological function of the colon. Most of the major chemical constituents of fibre are now identified and some are isolated in pure form (Southgate, 1976; Berk, 1976). Though the details have yet to be worked out, it is also known that dietary fibre undergoes bacterial fermentation in the colon with release of a number of chemical byproducts (Mangold, 1934). These mainly included low carbon atom compounds (e.g. VFA) and the gases  $\text{CO}_2$ ,  $\text{H}_2$  and  $\text{CH}_4$  (Prins, 1977; Stevens, 1978). The effects of dietary fibre on colon function are probably mediated through these metabolic products and the study of these substances could enhance understanding in the field. Though animal experiments can be designed so as to follow colonic fermentation in situ, in man the right colon, where most of the fermentation of dietary fibre takes place is not easily accessible and does not lend itself for manipulation. Intubation through the mouth is possible, but it is too involved for the subject to be readily acceptable. Moreover, the tube as a sampler is limited by the diameter of its lumen, the distance from the mouth to the colon and the consistency of the colonic contents. Patients with colostomy might be used, but the bacterial flora of the intestine in such situations is quite different from normal colon and the

stoma is exposed to atmospheric air making strict anaerobic metabolism difficult. Thus an indirect method of study is desirable. The metabolic byproducts (i.e. VFA and the gases) are either passed in faeces and flatus or absorbed into the portal blood circulation; VFA being mostly metabolised in the liver and the gases excreted by the lungs unchanged. The measurement of these substances in the stool, flatus and expired breath would be a suitable method of assessment of colonic fermentation (Calloway, 1968; Newman, 1974). Particularly the measurement in the breath of the two gases,  $H_2$  and  $CH_4$ , which are not produced by mammalian cell metabolism, can be a simple, non-invasive and unembarrassing procedure to indicate the level of dietary fibre fermentation in the colon.

Techniques of sampling and gas chromatographic detection of the small amounts in the breath, the behaviour of the gases in the intestinal lumen, the representativeness of the breath sample to the luminal concentration and some of the characteristics of breath excretion of  $H_2$  and  $CH_4$  have been investigated (Calloway, 1968; Levitt and Bond, 1970). Levitt and his co-workers, kept the subjects in air tight plastic hoods with closed circuit respiratory system and collected all the  $H_2$  and  $CH_4$  excreted in the breath over a period (Levitt, 1969; Levitt and Donaldson, 1970). This system despite its relative accuracy is cumbersome and unnecessarily complicated for the level of information it provides. It is also quite demanding to the subject. Calloway used a simple rebreathing technique into a multilaminated plastic bag, the purpose of which was to concentrate the  $H_2$  and  $CH_4$  in the expired air (Calloway and Murphy, 1968). Metz et al (1976a) used a single end expiratory sampling method based on the time honoured Haldane-Priestley (1905) alveolar sampling technique. These workers have also satisfactorily

verified their modified method of sampling against that of Levitt.

With respect to the behaviour of the gases in the intestinal lumen, investigations have demonstrated that most of the  $H_2$  and  $CH_4$  gas produced is absorbed into the blood stream and excreted by the lungs (Calloway and Murphy, 1968; Murray et al, 1976; Bond et al, 1978). A variable amount of gas may be passed as flatus, but the amount excreted in the breath is always proportional to the concentration of the specific gas in the intestinal lumen (Calloway, 1968; Levitt, 1969). Thus, measurement in the breath, though unable to quantitate all the gas evolved in the gut, is a simple and reliable indicator of the level produced.

In this project, the method of sampling of Metz et al (1976a) was adapted with certain modifications and after a pilot trial of the different methods available. Instead of the rubber tubing they employed, a polypropylene tube with the same dimension was used for simplicity of setting and further minimisation of gas leakage while sampling. A mouth piece was also fitted at one end (Fig. IIa). Gas leakage from the sampling syringe was tested over a variable time and environmental conditions. This showed that the syringe was sufficiently leak proof over 10-12 h in room conditions and this allowed the keeping of samples for some hours before processing. Though leakage rates found for longer periods of storage were within acceptable range, it was never necessary to keep the samples for analysis on a different day.

Available methods of detection of the two gases required separate gas chromatographic detectors; flame ionization for  $CH_4$  and thermal conductivity (Katharometer) for  $H_2$ . It was decided that a combined measurement of the two gases with one detector would be desirable and cheaper, and possibilities



towards this were investigated. By testing different variables which may affect the gas chromatographic separation of the two gases (i.e. packing, detector temperature, column temperature, carrier gas, flow rate of carrier gas, etc.) it was possible to choose suitable working conditions for the measurement of the gases together (Table IIa). This mainly involved changing the carrier gas to  $N_2$  (oxygen free), which eliminated the huge breath  $N_2$  peak that obliterated the breath  $CH_4$  peak. However, the katharometer was also found to be less sensitive to  $CH_4$  and this was overcome by manipulating the bridge current and detector temperature, the increasing of which gave improved sensitivity to  $CH_4$  (Figs. IIc and IId).

By attaching a small tube containing silica gel and soda lime to the sampling loop, water vapour and  $CO_2$  in the breath sample were prevented from entering the gas chromatograph, thus reducing the deterioration of the packing and detector filaments. The soda lime and silica gel were changed as required. The  $O_2$  in the breath was not easily removed, but it did not interfere with the satisfactory separation and detection of the desired gases.

The reliability of the response of the instrument was assessed in different ways. First, injection of different concentrations of the gases gave a linear response. Second, the injection of 20 consecutive samples with the same concentration of gases gave a coefficient of variation of less than 4%. Third, a series of 30 paired breath samples with a range of concentration of gases, gave a coefficient of variation of 1% for  $H_2$  and 7% for  $CH_4$ . These findings are satisfactory in light of the very small amount of the gases found in the breath, since a small variation would be magnified when expressed in percentage terms. Peak resolution

between  $H_2$  and  $O_2$  was calculated to be 3.3 and that between  $O_2$  and  $CH_4$  6.0. This degree of separation is well below the minimum peak resolution of 1.0 accepted in gas chromatographic analysis (Pattison, 1969).

Since smoking was found to affect the breath  $H_2$  and  $CH_4$  concentration, it was avoided during the test period in most instances and in those subjects where this was not possible at least a 10 min gap between smoking and sampling was allowed. A pilot test on the effect of smoking on breath sampling has demonstrated that 10 min was sufficient for the contaminant gas to wear off with normal breathing (Fig. IIg).

The human faecal bile acid analysis method used in this project was an established method in use in our laboratory and elsewhere and problems were not encountered. However problems arose in using the same method for the measurement of bile acids in the intestinal contents of the rat. This was because of the muricholic group of bile acids and the proportionally high concentration of fat in the intestinal contents. The presence in the muricholic bile acids of a hydroxyl group at C-6 close to that at C-7 led to breaking of the steroid ring during oxidising of the sample and the high concentration of fatty acids in the intestinal contents hindered adequate extraction of the bile acids. The problem was overcome by adapting other suitable methods. These included the further purification and extraction of the sample by using TLC and the formation of the TMS ether and methyl acetate derivatives instead of ketones for the GLC measurement. The reliability of both the methods and their precision were assessed by doing recovery experiments and by analysis of the samples in two analytical batches.



For the human bile acid method, the recovery rate of the conjugated cholic was found to be 70-80%, while that of the free bile acids 100%. The coefficient of variation for the same sample on different days was 2.5%. For the rat intestinal content method, the recovery rate was 80% and 97-100% respectively. Analysing 10 pairs (with a range of concentration) of the same samples on different days showed a coefficient of variation of 7.9% for the small intestinal samples and 12.7% for the colon. This level of recovery and analytical precision are quite acceptable for this type of analysis. Both methods appear to be less suited for the analysis of conjugated bile acids; but this in practice (i.e. measuring faecal bile acids) is not much of a disadvantage except when measuring bile acids from bile duct and small intestinal samples.

The fibre sources used were mainly acquired commercially (Appendix 1 and 2), and were allegedly of high purity and chemically undamaged. But, because of the nature of the extraction procedure available at the present some degree of chemical alteration from that naturally occurring in the intact plant may be expected during the isolation of the individual polymers (Southgate, 1976). Though this cannot be avoided, the detailed characterisation of the polymers would make them suitably standardised for investigative purposes. Hemicellulose was difficult to buy commercially and the small amount used here was graciously supplied by Charleston Research Centre. The purity and chemical integrity of the substance cannot be verified. The carrot was grown and cropped under experimental condition from one species and most of its properties were worked out in our laboratory (Robertson et al, 1979). Thus, interspecies difference and variation due to source of supply<sup>were</sup> eliminated. The

same applies to the bran, which was characterised and supplied by AACC. The usage of standardised or well characterised fibre sources, it is hoped, would help in making meaningful comparisons of results gathered from different laboratories. There is some confusion in reported studies because of usage of different sources of fibre with varying properties and extraction procedures.

The chemicals used in the different procedures were of analytical grade procured from different chemical firms (Appendix 4). Some of the chemicals were redistilled before usage to improve their purity.

The problem of how and when to administer the test substances and the frequency and duration of sampling the breath were decided first by studying the normal breath  $H_2$  and  $CH_4$  excretion pattern and then doing some trial tests to choose the most acceptable and convenient form of conducting the individual experiments. With respect to the normal excretion pattern of  $H_2$  and  $CH_4$  the details will be discussed in the next section. In summary, it was found that  $H_2$  and  $CH_4$  excretion levels in the breath were very low;  $H_2$  had a regular pattern of excretion, being high in the morning, decreasing to low values around mid-day, followed by some afternoon rise.  $CH_4$  excretion did not fluctuate in a regular manner during the day. The level of  $H_2$  excretion was reduced by fasting but  $CH_4$  remained unaffected. The variation of  $H_2$  and  $CH_4$  levels in the breath within an hour was minimal. From the pilot trials with the test materials it was decided to administer the fibre sources uncooked to avoid the uncertainty of the effects of heat during cooking. Carrot was given raw and the individual polymers mixed with water and black currant juice as flavouring. The response to most of the polymers was detected within two hours and by about six hours most of it was

complete. Thus, the administration of the test substances in the morning after overnight fasting, and following the breath sample hourly during the day for 6-10 hours, while subjects were still fasted, was chosen as the most suitable method of investigation.

With respect to the subjects, of the volunteers who participated in most of the investigation in this project, half were members of staff and half students. This to some extent makes them a select group in that the staff through their awareness of the nature of the research would be expected to be motivated and possibly biased. The students may not be representative of the community at least in their eating habits. The sex distribution was good but of necessity there was a bias to the younger age group and results must be interpreted in light of this.

#### Normal Breath Hydrogen and Methane Excretion

The mean breath  $H_2$  excretion was below 0.50  $\mu\text{mol/l}$  and this low grade excretion was further reduced by fasting overnight (Fig. IIIa). The random sampling of breath in the community at Pilton, North Edinburgh, also affirmed the low level of  $H_2$  excretion in the normal situation (mean = 0.25  $\mu\text{mol/l}$ ; range 0 - 1.07  $\mu\text{mol/l}$ ). In fact only two of the seventy odd samples were found to be above 0.90  $\mu\text{mol/l}$ . These results further confirm previous reports which showed low levels of normal breath  $H_2$  excretion in populations eating a European type of diet (Calloway and Murphy, 1968; Levitt and Ingelfinger, 1968; Metz et al, 1976a).

In this study an attempt was made to examine the normal excretion pattern of  $H_2$  in more detail by frequent sampling of breath during the day. Analysis of the results showed a relatively high level of excretion early in the morning, a decrease in the level around mid-day and some rise early in the afternoon (Figs. IIIa and IIIb).

The fluctuation of  $H_2$  excretion appears to be related to the food eaten during the previous hours. The early morning raised values are probably due to the previous evening meal and the afternoon rise is due to breakfast. This suggestion is supported by:-

1. The decrease of the morning breath  $H_2$  rise by a long period of fasting or by light evening meals.
2. The abolishing of the afternoon rise by fasting during the day (i.e. avoiding breakfast).

This pattern of excretion was maintained in individuals tested on different days or on consecutive days indicating the regularity of eating habits which people follow (Fig. IIIc).

Breath  $CH_4$ , on the other hand, was found to be individual, with two population groups, one of excretors and the other non-excretors. In those who excreted methane, excretion level was unaffected by fasting and there appears to be no obvious regular daily variation (Figs. IIIb and IIIId). Levitt and Bond (1970) have reported that a third of the population were methane excretors and the remaining two-thirds non-excretors. Calloway's (1968) experience was similar. This is also supported by the findings in the relatively young age group studied (Fig. IIIe). About a third of this group were found to be excretors. But, in the community group the usually expected ratio of excretors and non-excretors was not seen. This finding was unexpected and raised question as to its cause. The results were then further scrutinised and factors which might have contributed to the difference were looked into. No association was found between sex and  $CH_4$  excretion or disease state and  $CH_4$  excretion. When the breath  $CH_4$  excretion values were arranged into age groups, it was found that the percentage of excretors increased with age. Moreover, in those who excrete  $CH_4$ , the level



of excretion tended to be higher with age (Fig. IIIe).

The reasons behind the individual difference in  $\text{CH}_4$  excretion are obscure and unresolved. Some of the suggestions which have been forwarded include differences in dietary habit, colonic bacterial flora or genetic make up (Levitt and Bond, 1970; Bond et al, 1971). None of the experimental evidences available to date clearly support any one of these factors fully. Contrary to the dietary hypothesis, it has been shown that people who are fed the same kind of diet either at home or in an institution, still reflect the same  $\text{CH}_4$  excretion pattern as a random population does. With respect to the bacterial hypothesis, studies on subjects who occupy the same environment or are intimately related and who might be expected to harbour the same type of bacterial flora do not fall exclusively into the same category of excretors or non-excretors. As to the genetic proposition, even though there is not sufficient data, the few available studies on siblings do not clarify this hypothesis fully.

In these preliminary studies, it was found that  $\text{CH}_4$  excretion was not influenced by meals, dietary fibre intake or the administration of a number of dietary fibre carbohydrates (Figs. IVb and IVc). This does not tally with the dietary hypothesis, yet it does not rule out the role of other dietary constituents like protein or fat. Calloway et al (1966) studying the evolution of gases by intestinal bacteria in vitro cultures of colonic digesta, found that when the digesta was cultured with different substrates more methane was formed with amino acid substrates as compared to carbohydrates. This result is strong support for the dietary hypothesis but still falls short of explaining the persistent observation of two groups, producers and non-producers of methane, despite similar dietary

intake. The increase in the number of excretors with age found here would suggest other factors than diet. One likely factor is the bacterial flora of the gut. It has been shown by a number of investigators that the faecal flora changes with age (Gorbach et al, 1967; Borriello et al, 1978). Older people tend to harbour fewer anaerobic lactobacilli and greater numbers of coliforms and fungi, when compared with younger counterparts. Though there is no reported investigation which has attempted to study the distribution of methanogenic bacteria with age, it is quite possible that the older individuals may be harbouring more of this bacterial group. Another factor to consider, particularly in the group investigated here, is the environment. The 68 people studied came from one area of a general practice and a common environmental factor may account for the difference in the result. The most easily accessible variable in the environment was the methane level in the room air. This was measured both from the subjects' home and the laboratory. There was no obvious peculiarity observed and the level was low with minimal variation.

Methane is thought to be formed during reduction of  $\text{CO}_2$  with  $\text{H}_2$  and formic acid, by a special group of fastidious anaerobic bacteria known as methanogenic bacteria (Wolfe, 1971; Prins, 1977). Only one species of the group has been isolated from faeces of man (Nottingham and Hungate, 1968). These bacteria are also considered to be totally dependent on the presence of other anaerobic organisms producing  $\text{H}_2$  and formic acid (Clarke, 1977). If such is the case, it would be expected that  $\text{CH}_4$  formation is related to  $\text{H}_2$  formation. There is experimental confirmation to this effect from studies in ruminants and selective cultures of the specific bacteria (Hungate, 1966; Cole, 1976). Both gases are produced in the rumen and

respond in concert to added exogenous materials (Mangold, 1934). Methane production by bacterial cultures in vitro could be enhanced by the addition of external  $H_2$  (Prins, 1977; Sauer et al, 1977). The results in this study did not show any relationship between the two gases. This finding is also in agreement with other reports (Calloway, 1968; Levitt and Bond, 1970). It seems, therefore, that the factors influencing  $CH_4$  formation in man are more complex and different from those in ruminants or in vitro cultures. It is possible that a number of factors are working together to bring about  $CH_4$  formation and a single factor such as diet or bacteria may not be the sole determinant.

From the findings on breath  $CH_4$  excretion in the present study, it seems more appropriate to divide the population into three groups instead of two. There are three distinct groups encountered - those who are non-excretors at all times (below 0.10  $\mu\text{mol/l}$ ), those who produce a modest amount most of the time (around 0.50  $\mu\text{mol/l}$ ) and those who excrete very high levels (greater than 1.00  $\mu\text{mol/l}$ ).

In the preliminary investigation here no attempt was made to control diet and the dietary history did not determine the type or the chemical composition of the dietary fibre. Thus, the amount and source of the fibre ingested is bound to vary amongst the subjects and the total fibre estimated from the dietary history does not necessarily reflect the potential for  $H_2$  production in the gut. Different sources of fibre give variable amounts and pattern of gas production. Ingestion of beans is followed by high levels of gas production, while bran results in modest amounts (Calloway, 1966; Meyer and Calloway, 1977). Large volumes of  $CO_2$  and small amounts of  $H_2$  are produced after consumption of prunes but the converse is true for raisins (Hickey et al, 1972). Hence, the lack

of significant correlation between the dietary fibre intake and the breath excretion of  $H_2$  or  $CH_4$  was possibly due to the differences in the chemical composition of the fibre source consumed by the individual subject. Moreover, retrospective dietary history is not a very reliable method for the accurate assessment of nutritional intake and too much weight should not be attached to this aspect of the result.

To summarise, the important observations from these set of experiments were:-

1. Breath  $H_2$  excretion follows a regular pattern depending on diet and is easily manipulated by the administration of substrates, lending itself as a useful indicator of colonic fermentation.
2. Methane excretion is individual with no obvious daily variation and the level unaffected by diet.
3. Methane is not produced by all individuals and its excretion appears to increase with age possibly due to bacterial flora changes.

#### Mouth-to-Caecum Transit Time

The mouth-to-caecum transit time (MCTT) was measured in order to determine the time taken by a test meal to reach the caecum. During the trial an interesting observation emerged. Lactulose and raffinose when administered in the same amount and form, had different transit (or  $H_2$  production) times. This phenomenon was thought worthwhile to investigate further.

Various factors might influence the transit time of food through the gut. The type of food, its consistency, osmolality, its viscosity, volume, the myo-electric activity of the wall, intra-luminal events, hormones and emotional factors are amongst the important ones (Hunt and Knox, 1968; Weisbrodt, 1974; Bueno et al,



1975; Bond and Levitt, 1975; Jenkins et al, 1978; Cummings, 1978a). Each of these factors may have different roles and degrees of influence at different parts of the gut (i.e. stomach, small intestine, caecum and colon) or even functional sections of a part (Dillard et al, 1965; Wiggins and Cummings, 1976). Gastric emptying is influenced mainly by texture, osmolality and the chemical nature of the meal. Solids and fatty foods leave the stomach slowly, whilst liquids and carbohydrates have a more rapid emptying time (Hunt and Knox, 1968; Clemens et al, 1975). Dietary fibre may increase gastric emptying (McCance et al, 1953) or decrease it (Heading et al, 1976). Colon transit is mainly dependent on the bulk of the residue, metabolic products of bacteria and to some extent on social factors (Connell and Smith, 1974; Spiller et al, 1977; Cummings, 1978). Small intestinal transit is comparatively short, but factors which influence it are not well studied (Clemens et al, 1975; Bond and Levitt, 1975; Losowsky, 1978). Dillard et al (1965) studying the volume-flow relationship during the transit of fluid along the human small intestine found that the small intestine handled an increase in volume flow by distending, without changing the transit time. This was found only to a limited extent, i.e. 3-7ml/min, but beyond 7ml/min the increase in volume is handled by shortened transit time. They also estimated that the transit time through a 100 cm segment of jejunum to be about 31 min and ileum about 19 min. From these values it can be calculated that the mean transit time of an isotonic solution along the whole small intestine to be of the order of 80 min. Other reports using barium and breath  $H_2$  are not very different from this value (Kim, 1968; Bond and Levitt, 1975; Metz et al, 1976b).

In this particular study most of the controllable variables

expected to affect the mouth-to-caecum transit time were kept as near constant as possible and the effects of changes of osmolality and molecular weight of the oligosaccharides assessed separately. The test meals were of similar consistency (i.e. liquid), containing in many ways similar carbohydrates of equal weight (10g) and the viscosity of the meals were comparable. The expected difference and the actual time course of gastric emptying should be minimal, since liquids leave the stomach very rapidly and in exponential fashion. The transit time measured is effectively the small intestinal transit time (SITT) (Bond and Levitt, 1975).

It was shown that the three oligosaccharides tested had significantly different MCTT (alias SITT), increasing with increased molecular weight (Table III d). Several factors may account for the difference, but two appear to be most important. First, the difference may be a true reflection of the time taken by the oligosaccharides to traverse the small intestine. Alternatively, the difference may be due to a difference in the rate of bacterial metabolism of the oligosaccharides once they reach the caecum. If oligosaccharides of different molecular weight pass along the intestinal tract at different rates, what then is the physical and chemical basis for it? Viscosity has been implicated in influencing transit time of dietary fibre sources along the small intestine (Jenkins et al, 1978). But, in this study there was no significant difference in viscosity between the test meals. Osmolality differences was thought to be important since osmolality is known to influence gastric emptying (Hunt and Knox, 1968). But changing the osmolality of the same oligosaccharides by half was not followed by change in transit time. Neither did different oligosaccharides with comparable osmolality have the same transit time (Table III d).

Another tempting possibility is the difference in molecular size, bigger molecules moving slower than smaller molecules, but a subtle difference of the order found between these oligosaccharides can hardly account for the big transit difference observed. A more likely possibility is the amount of water bound to the different oligosaccharide molecules (Kramer, 1953). This of necessity would assume a decrease of water bound with increasing molecular size for which there is no experimental evidence.

Bond and Levitt (1975) by direct instillation of lactulose into the caecum of man have demonstrated a time delay between instillation and  $H_2$  evolution of about five minutes. Similar measurements for raffinose and stachyose are not available. But, in vitro, studies with anaerobic bacterial cultures isolated from dog colon and using different sugars as substrates, showed a slower rate of fermentation for stachyose and raffinose as compared to glucose (Rackis et al, 1970). The authors suggested that the difference was due to the time needed to break the oligosaccharides into monosaccharides before  $H_2$  production by the bacterial cell can commence. Surprisingly, they also showed differences in the rate of gas evolution between different monosaccharides and the values for raffinose and stachyose were of the same order. Thus, their suggestion cannot fully account for the difference between the monosaccharides. Assuming the difference in the apparent transit time observed in this study may be a result of the extracellular breakdown of the oligosaccharides before absorption, the time difference observed which was of the order of about an hour can hardly be accounted on the basis of chain length difference, which is only one saccharide. Bacteria can absorb some disaccharides intact and the short time observed by Bond and Levitt (1975) for  $H_2$  evolution when using lactulose supports the

possibility of absorption without extracellular hydrolysis.

The observation may further be clarified by direct instillation of the different oligosaccharides into the caecum of normal people or stoma of people with a colostomy. Other oligosaccharides of larger molecular weight were not tested because of rarity of the pure forms and the very high cost of separation or purchase. Only two tests were performed with stachyose for the same reason (one test requiring 10g stachyose cost about £100). However, from observation in the other set of experiments in this project, the polysaccharide hemicellulose, which has a much greater molecular weight compared to the three oligosaccharides, did not show a prolonged time for the breath  $H_2$  to increase as would have been expected.

The lack of correlations between whole gut transit time (as assessed by the SST) and mouth-to-caecum transit time (as assessed by breath  $H_2$ ), and also between dietary fibre intake and MCTT, while there was correlation between dietary fibre intake and whole gut transit time, suggests that different factors are involved in influencing these two variables.

In conclusion, the MCTT varies individually and is dependent on the molecular weight of the oligosaccharide used. The osmolality of the oligosaccharide solution used and the habitual dietary fibre intake appear to have no influence.

#### Metabolism of Dietary Fibre Components

The investigation on the acute administration of different chemical components of dietary fibre showed that few of the different polymers when ingested on a single occasion resulted in an enhanced production of  $H_2$  or  $CH_4$ . This might suggest that the polymers are not being metabolised at all. Alternatively, they may have been metabolised along other pathways not involving  $H_2$  or  $CH_4$  formation. Partial



degradation of carbohydrates to VFA and other short chain carbon molecules is well recognised in anaerobic bacterial metabolism (Prins, 1977). In such situations the  $H^+$  ion produced during phosphorylation may combine with other organic compounds formed as intermediates. Thus, the apparent lack of evidence for the breakdown of some of the component polymers may be due to the limitations of the method used. Also, the study period of 8 hours may not have been adequate for the bacteria to ferment some of the chemicals, but prolonged follow up of some of the individuals for 10-12 hours render no delayed  $H_2$  and  $CH_4$  increase.

Another important factor to consider is the bacteria in the caecum. Individuals have subtle differences in the bacterial flora they harbour in their gut and dietary habit has some part to play in this (Gorbach et al, 1967; Moore et al, 1978). Though short term dietary changes are not accompanied by changes in faecal flora, long-term changes may do so (Golden and Gorbach, 1977; Finegold and Sutter, 1978). If differences in bacterial flora between the individuals was a factor in this study, the response to the different polymers could not have been consistent in all the subjects. However, the substances were not administered for a sufficiently long period to bring about changes in bacterial flora, with establishment of a species with the appropriate enzyme system to hydrolyse the particular polymer. The effect of long-term administration, though not tested with the individual components, was looked into with other sources of fibre and shall be discussed in the appropriate section.

Looking at the results for the individual polymers, starch did not increase breath  $H_2$  and  $CH_4$ , presumably due to complete hydrolysis by  $\alpha$ -amylase and absorption of the hydrolysis products

at the upper part of the small intestine. Raw starch has been shown to be less hydrolysed in the small intestine and some of it may reach the caecum (Sandstedt and Gates, 1954). The amount administered in this study (20g) is unlikely to saturate or inhibit the enzyme activity so that a sufficient amount reaches the caecum to give an observable effect. If at all, starch seems to have depressed base line  $H_2$  production (Fig. IVa). Lignin is reputed to be resistant to hydrolysis by animal and bacterial enzymes which is consistent with the result here. On the other hand, pectin, which is reported to be completely hydrolysed, was expected to result in an increased  $H_2$  and  $CH_4$  excretion (Werch and Ivy, 1941; Werch et al, 1942; Southgate and Durnin, 1970). This was not the case and it may be due to hydrolysis along other pathways which did not lead to  $H_2$  formation. Partial degradation to VFA is one such pathway, but still some  $H_2$  or  $CH_4$  formation would have been expected during the change of the partial degraded carbohydrate to VFA (Wolfe, 1971; Prins, 1977). Other organic substances may have accepted the "free" electrons, but there seems to be no obvious reason why bacteria capable of forming  $H_2$  (as indicated by the other polymers) would prefer other modes of disposing the 'excess' electrons when it comes to pectin. Perhaps, a more likely explanation for this finding may be the possible presence of a specific group of bacteria which metabolise pectin alone, through a pathway leading to end products other than  $H_2$  and  $CH_4$ . The modest production of  $H_2$  during hemicellulose administration may be due to the comparatively small amount used (10g), yet the gas excretion is not comparable to 10g raffinose.

Though lactulose, raffinose, stachyose and hemicellulose were accompanied with high levels of  $H_2$  production, the  $CH_4$  excretion was

unaffected and remained individual. This has been the experience of other investigators too (Calloway and Murphy, 1968; Levitt and Ingelfinger, 1968), but what is difficult to explain is the lack of increased (or decreased) production by the individuals who were found to be  $\text{CH}_4$  excretors. This suggests that the substrate for  $\text{CH}_4$  formation is something other than carbohydrates and its formation is unrelated to  $\text{H}_2$  formation. In vitro experiments do not support this view. It has been shown that mixed cultures from the colon produced both  $\text{H}_2$  and  $\text{CH}_4$  from carbohydrate substrates including raffinose and stachyose (Calloway et al, 1966; Rackis et al, 1970). The formation of the two gases is also inter-related (Wolfe, 1971; Hungate, 1976; Prins, 1977). It appears, therefore, that the factors influencing  $\text{CH}_4$  production in man are multifactorial and complex and as such not readily altered.

The physical nature of the polymer may be a factor affecting bacterial hydrolysis as discussed earlier. This aspect was looked into, particularly with cellulose and pectin. For these two polymers, differences in physical property (i.e. particle size) and some chemical change in the molecule appear to make no appreciable change in the  $\text{H}_2$  or  $\text{CH}_4$  excretion pattern. On this point, one can only be certain about the substances affecting  $\text{H}_2$  formation, but for those polymers possibly metabolised through other pathways breath  $\text{H}_2$  and  $\text{CH}_4$  will not reflect the difference.

In summary, acute administration of lactulose, raffinose, stachyose and hemicellulose increase  $\text{H}_2$  production, while cellulose, pectin and lignin do not. Methane production is individual and unaffected by any of the polymers. Differences in physical properties of the same chemical appear to have no influence on the  $\text{H}_2$  or  $\text{CH}_4$  production.

### Acute and Long-Term Administration of Carrot

The substantial absorption capacity for water, cations and bile acids and the provision of a suitable matrix by carrot fibre, would be expected to provide a suitable media for bacterial activity. The results from the long-term administration of carrot in this study confirm the expectation. Carrot fibre was hydrolysed by colonic bacteria producing  $H_2$  but not  $CH_4$ . The results also suggest that the metabolism of carrot fibre does not start instantly but takes time till the appropriate flora probably establish themselves. This proposition is supported by the lack of significant increase in breath  $H_2$  following the acute administration (Table Va) and the first week of the chronic administration, while there was a significant increase during the second and third weeks (Table Vc). Thus, the change in breath  $H_2$  excretion is probably related to change in the bacterial ecosystem or metabolism and this apparently takes time to readjust. The modest increase in breath  $H_2$  must have been due to the comparatively small amount of fibre content (about 8g) in the 200g carrot ingested. Once again there was no effect on  $CH_4$  excretion (Fig. Vd).

Though the high bile acid adsorption capacity of carrot may be the explanation for the increase in faecal bile acid excretion, it is also possible that it was due to change in bacterial flora or metabolism. This view is supported by the sustained high excretion of faecal bile acids despite the cessation of carrot intake (Table Ve). However, there ~~was~~ a proportionally higher excretion of primary bile acids during carrot intake and this indicates a greater effect of adsorption rather than <sup>of</sup> bacteria. Paradoxically, the same proportional relationship between the primary and secondary bile acids excreted was maintained during the period when carrot intake was withdrawn. This has also been observed with bran administration



(Eastwood et al, 1973; Kay and Truswell, 1977).

The quantity of dry matter in the 200g carrot ingested was very small (about 7g) and some of this would have been metabolised. However, carrot fibre has a high water holding capacity of the order of 23.4 g/g (McConnell et al, 1974). This might explain the increase in wet stool weight while there was little change in the dry weight (Table Vf). There was also no significant change in the whole gut transit time. Cummings et al (1978) using carrot fibre extracts have found similar results for the transit time. This would suggest factors other than water bulking ~~to~~ be responsible for the speed of transit of residue in the colon. Volatile fatty acids, pentosans and other bacterial metabolites have been considered as the cause for the decrease in transit time during high fibre intake (Williams and Olmsted, 1936; Cummings et al, 1978; Cummings, 1978a).

Different sources of dietary fibre have been shown to have different effects on serum cholesterol. Pectin has been shown to decrease serum cholesterol whilst bran has no effect (Keys et al, 1961; Eastwood, 1969; Durrington et al, 1976). The decrease in serum cholesterol whilst eating carrot, is not easily explainable. Carrot does not contain a high amount of pectin and so cannot be the cause for the decrease (Southgate et al, 1976a). Either carrot fibre acts as a physical adsorber of cholesterol and its metabolic products, increasing the excretion in faeces, or the effect on serum cholesterol was secondary to alterations in the bacterial degradation of cholesterol in the colon as a result of carrot ingestion.

In conclusion, raw carrot fibre is fermented in the colon with associated  $H_2$  production and no effect on  $CH_4$  evolution. There is a lag period of a few days between start of carrot ingestion and increase in breath  $H_2$  excretion. Carrot fibre appears to influence

serum lipids and faecal bile acids with an aftermath effect. In the amount administered here, carrot's effect on stool weight is modest and it makes no impact on the whole gut transit time.

#### Effects of Dietary Fibre on Bile Acid Metabolism in the Rat

More than 95% of the total bile acid pool in the rat's body is found in the intestinal tract (Fisher et al, 1976; Uchida et al, 1978). The distribution along the tract will depend on the rate of absorption, the transit time and diet (Bergstrom and Danielsson, 1968). The three variables are also interdependent with each other. In this study it was found that the amount of total bile acids in the small intestine was greater with high fibre diet as compared to the low fibre diet (Figs. Vb and Vc). Several factors may account for this. In the first place, the small intestinal dry content was greater on the high than on the low fibre dietary regimes. This may be the reason for the high concentration of bile acids in the rats fed with high fibre containing diets. In addition, the high fibre diet may entrap and/or adsorb bile acids in its mesh leaving less to be reabsorbed (Eastwood and Boyd, 1967). Alternatively, more bile acids may be secreted to the small intestine during the high fibre dietary regimes increasing the total small intestinal concentration at a given time. This explanation is supported by the increased liver cholesterol 7 $\alpha$ -hydroxylase activity during this period (Fig. Vg). All the factors, however, are not mutually exclusive and each may contribute a part.

In the colon the converse was observed, i.e. the total colonic bile acid was greater with fibre depleted than on the high fibre diets (Fig. Vb). This finding is difficult to explain, since the same trend as that of the small intestine would be expected. One possible explanation may be the difference in transit time of

colonic contents when the rats are on the two dietary regimes. Gustafsson and Norman (1969), using carmine as a faecal marker, have shown an increase in transit time in rats fed on low fibre semisynthetic diet as compared with those fed on high fibre containing chow pellet. Thus, the high fibre containing diet by accelerating transit will enhance faecal excretion of bile acids, while <sup>the</sup> fibre depleted diet retards evacuation and favours accumulation of bile acids in the colon. If bile acids are retained in the colon for a longer period, the chances for bacterial action increase and more degradation products may be formed. In this study, there was a considerable increase in the relative proportion of bile acids of bacterial transformation, in the rats fed on the fibre depleted diet (Fig. Vf). With the decrease of transit, more of the changed bile acids may also be absorbed into the enterohepatic circulation and have a negative feedback effect on bile acid synthesis in the liver (Bergstrom and Danielsson, 1968; Danielsson, 1973b). The decrease of liver cholesterol 7 $\alpha$ -hydroxylase activity was possibly a result of this effect (Fig. Vg).

The lack of effect of the different dietary regimes on the total intestinal bile acid pool was an interesting finding. Some workers who have looked into this aspect of bile acid metabolism in the rat, have reported a decrease in the pool size in rats fed on low fibre semisynthetic diet (Gustafsson and Norman, 1969; Fisher et al, 1976). In this present investigation, though there was a large difference in the total bile acid concentration on the two types of diet, both in the small intestine and the colon, the total intestinal bile acid pool (i.e. S.I. + C.) remained unaltered. This apparent anomaly may be accounted for by the difference in methodology. Gustafsson and Norman (1969) estimated the pool size by administering (24-<sup>14</sup>C)

cholic acid and measuring the radioactivity of cholic acid and its metabolites in the small intestine and colon separately. They reported an increase in the total bile acid pool on pellet diet and no significant effect of dietary fibre content of the diet on the caecal and colonic concentrations. Fisher et al (1976), measured most of the bile acid components except  $\alpha$ - and  $\mu$ -muricholic acids in the intestinal content as a whole. The result showed about 40% decrease in the total bile acid pool, when rats were fed on low fibre diet as compared to those on high fibre containing pellet. No qualitative change in the bile acid pattern was observed on the different dietary regime. Looking at the results in this study, about 60% of the total intestinal bile acid pool in the rats fed with fibre depleted diet and 30% in those fed with high fibre containing diets, is made up of the muricholic group of bile acids, i.e.  $\alpha$ -,  $\beta$ - and  $\mu$ -muricholids (Tables Vi and Vj). Similar findings have also been reported by a number of investigators recently. Madsen et al (1976) showed that more than 50% of the faecal bile acids in rats on regular chow pellet, consisted of hydoxycholic acid and  $\mu$ -muricholic bile acids alone. Eyssen et al (1977), studying the bile acid pattern in male and female rats have indicated the predominance of the muricholic group of bile acids in the male rats. Though, Uchida et al (1978) in studying the distribution of bile acids in the rat, were unable to specify the bile acid moiety, more than 40% of the colonic bile acids ran as an unidentified peak. This unidentified peak appears to be  $\mu$ -muricholic acid. If the results of the two authors who reported a decrease in the bile acid pool are examined, it will be apparent that Gustafsson and Norman did not measure muricholic bile acids. Fisher and colleagues, on the other hand, showed that 70% of the



total bile acids in the intestinal tract consisted of cholic and deoxycholic acids alone, while  $\beta$ -muricholic and hyodeoxycholic acids accounted for about 8% each. Thus, there is a strong evidence indicating that the finding of decreased bile acid pool by the two investigators may have been due to the failure to measure this important group of bile acids in the rat.

About 20  $\mu\text{mol}/100\text{g}$  body weight was found to be the total intestinal pool, in the rats fed on commercial chow pellet. This value is comparable to most reported values (Norman and Sjoval, 1958; Weiner and Lack, 1968; Gustafsson and Norman, 1969), but is much less than a few recent reports (Fisher et al, 1976; Uchida et al, 1978). This is rather puzzling because if anything a higher bile acid result than those reported up to now would have been expected as a result of measuring all the muricholic group of bile acids. The only possible explanation is that the workers though not qualitatively differentiating all the muricholic acids, may have been measuring them together with the identified peaks. The reported range of values are wide (10 - 60  $\mu\text{mol}/100\text{g}$ ) and the concentrations measured in this study are on the higher side of the values reported by most workers, yet the difference with the recent reports is unaccountable.

From the relative proportions of the individual bile acids in the small intestine, the changes of hyodeoxycholic acid were remarkable. Hyodeoxycholic acid concentration was greater on high fibre diets than on low fibre diet (Tables Vi and Vj). This is understandable, because hyodeoxycholic acid is thought to be formed from lithocholic acid, which in turn is formed from chenodeoxycholic acid and both of which showed a tendency to decrease with the fibre depleted diet (Tables Vi and Vj). Presumably the decrease in the chenodeoxycholic acid is related to the decrease in its formation in

the liver as a result of enhanced enterohepatic recirculation of bile acids in the rats fed with low fibre diet.

The exact inter-relationship of the different muricholic acids with the other bile acid moieties is not clear. It is probable that all are bacterial and hepatic products from primary and secondary bile acids (Danielsson, 1973a). Hence, the proportional increase, particularly that of  $\mu$ -muricholic acid, both in the small intestine and colon of the rats on low fibre containing diet, may be related to the increased chance for bacterial activity and intestinal absorption as a result of decreased transit time through the colon.

The decrease in the concentration of deoxycholic and lithocholic with the low fibre diet, though not quantitatively remarkable, must be related to the decrease of the precursors - cholic and chenodeoxycholic acids.

Most of the changes that accompanied the feeding of wheat bran supplemented soft diet, though similar to those of the commercial chow pellet diet, were less marked (Table Vi). This is probably due to two related factors. First, there was difference in the amount of fibre content in the two dietary regimes. The commercial chow pellet contained about 30% more of acid detergent fibre when compared with the bran supplemented soft diet (Table Vh). Second, though the lignin content was similar for both diets, other qualitative differences in the fibre polymers cannot be ruled out. So, the variation in the amount of bile acids observed could be a result of the difference in total fibre content, while the qualitative difference may be due to difference in composition and chemical property of the two dietary fibre sources. Moreover, in vitro and in vivo investigations in man indicate that wheat bran is a relatively poor binder of bile acids (Eastwood and Hamilton, 1968;

Story and Kritchevsky, 1976; Kay and Truswell, 1977). Thus, the qualitative and quantitative differences of the two dietary fibre sources may have played an important role in the shaping of the bile acid pattern observed in this study. These same factors may also account for the distinct difference in the liver cholesterol 7 $\alpha$ -hydroxylase activity of the rats on the two high fibre containing diets (Fig. Vg). The other subtle differences found but which cannot be generalised could be due to other minor qualitative differences in the other dietary constituents of the diets used (Table Vh). These dietary differences may have acted directly or indirectly by changing bacterial metabolism.

In summary, dietary fibre affects the metabolism of bile acids in the intestinal tract and its formation in the liver. This effect appears to be mediated through the physical properties of fibre which alters the distribution of bile acids along the intestinal tract and their excretion in the faeces. The colon, particularly in the rat, seems to play an important role in the qualitative changes of bile acids and the consequent effect on the synthesis in the liver. The different variables in bile acid metabolism i.e. bile acid synthesis in the liver, adsorption to dietary fibre, absorption into the enterohepatic circulation and colonic bacterial metabolism, should not be looked in isolation but as part of the whole process, each playing its role and at the same time intricately linked with the others.

CHAPTER VII

C O N C L U S I O N S



### CONCLUSIONS

The metabolism (fermentation) of dietary fibre by bacteria in the human colon may be studied by measuring gaseous metabolic end products excreted in the breath. Theoretically, the gases  $H_2$  and  $CH_4$  are most suited to study but in practice the value of  $CH_4$  as an indicator is somewhat limited. This is because it is only formed in some individuals and is resistant to manipulation.  $H_2$ , on the other hand, is produced by all individuals and has a regular pattern of excretion. It also responds sensitively and reliably to specific dietary alterations.

The study of the individual isolates of dietary fibre components showed that few of the polymers, in the manner and conditions investigated in this project, were metabolised through metabolic pathways which produced  $H_2$ . Of the different chemical polymers of fibre, it is the oligosaccharides (i.e. raffinose and stachyose) which produced the most  $H_2$ ; the polysaccharides and lignin were either unfermented or were partially degraded to other unmeasured compounds. Since with some of the polymers there was a subjective increase in intestinal gas it would be assumed that other volatile substances were being formed. The inclusion of these compounds as part of the measurement in such studies may be a useful addition. Carrot was metabolised in the colon producing  $H_2$ , but there was a delay before an increase in breath  $H_2$  was detected, suggesting change in faecal flora or metabolism. The lack of increase in breath  $H_2$  excretion when some of the polymers were administered may be due to the insufficient time for these readjustments to take place. Yet, the increase in breath  $H_2$  with oligosaccharides was instantaneous in all the subjects studied.

The effects of dietary fibre ingestion on colon functions are

related to its physical and chemical properties and may be mediated through the bacterial fermentation products and other organic and inorganic substances of the gut which are handled by the luminal bacteria. As was well illustrated by the rat experiment and supported by the findings in the carrot study in man, the change in the distribution of substances along the gastrointestinal tract following fibre ingestion is not only of physical consequence but is accompanied by profound metabolic changes and adjustment in the bacterial activity.

What emerged when the different results in this project were analysed was the deficiency in information. The design of some of the experiments were so dictated by acceptability and practical constraints that they suffered from superficiality. The measurement of VFA and  $\text{CO}_2$  would have been desirable extra information, particularly since they may have helped to verify the fate of the polymers which did not increase breath  $\text{H}_2$  excretion. The correct assessment of these variables are not simple since VFA may be absorbed into the portal circulation and metabolised in the liver before detection in the breath, while  $\text{CO}_2$  is exchanged with cellular sources and cannot be distinguished unless precursors are radioactively labelled. The lack of measurement of  $\text{H}_2$  and  $\text{CH}_4$  in flatus was another drawback. Its measurement together with an assessment of respiratory parameters like minute volume, for each individual participant, would have further facilitated the calculation of actual amounts of gas produced and thus improved accuracy. So would have the taking of more frequent breath samples.

In light of the different results, the experience gathered during the project and the weaknesses in the present studies, it

would be worthwhile to pursue further research in the following lines:

- (1) Administration of the different polymers singularly and in combinations for longer periods to metabolically controlled subjects and measuring breath  $H_2$  and  $CH_4$ , flatus gases, VFA in stool and the rest of the parameters of colon function.
- (2) Investigation of direct effects of bacterial fermentation products on the absorptive, secretory and motor activities of the colon. This would be best studied in animals.
- (3) Looking in more detail into factors which influence  $CH_4$  formation in man. The study of colonic bacteria and other dietary constituents would be profitable.
- (4) The study of other trace metabolic products excreted in breath and their use for diagnostic and research purposes.

CHAPTER VIII

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CHAPTER IX

A P P E N D I X

# Appendix 1

## Physico-Chemical Properties of Oligosaccharides Used

Properties	LACTULOSE		RAFFINOSE	STACHYOSE
	50% Solution	Pure		
Molecular Weight	342	342	594	738
Colour	Yellow	White	White	White
Texture	Syrup	Crystalline	Crystalline	Powder
Test	Sweet	Indifferent	Indifferent	Indifferent
Melting Point (°C)	-	173-178	78-82	100-104
Solubility (H <sub>2</sub> O)	Soluble	Soluble	Soluble (1:7)	Soluble
Ash (%)	-	-	0.1%	-
Moisture (%)	50	-	15	-
Source	Synthetic	Synthetic	Australian Manna Cotton Seed	Artichokes
Others	Galactose 8% Lactose 5%	-	Lead - 0.001%	-
Supplier	Duphar Lab. Ltd. Southampton, U.K.	Sigma Chem. Co. Ltd. Poole, Dorset, U.K.	BDH Chemicals Dorset, U.K.	Wessex Biochemicals Ltd., Bournemouth, U.K.

## Appendix 2

### Physico-Chemical Properties of Polysaccharides and Lignin Used

PROPERTIES	CELLULOSE				HEMICELLULOSE
	AV - 101	AV - 102	SW - 40	Na-COOH-ME (7HF)	
Fibre Length	50 $\mu$ m	100 $\mu$ m	140 $\mu$ m	40 mesh	-
Colour	White	White	White	Light cream	Brown
Texture	Powder	Powder	Soft Flakes	Powder	Powder
Solubility (H <sub>2</sub> O)	Insoluble	Insoluble	Soluble	Soluble	Suspension
Ash (%)	0.004	0.004	0.1-0.2	-	1.9
Moisture (%)	< 5.0	< 5.0	5.7	5 - 10	?
Heavy Metals (%)	< 0.001	< 0.001	< 0.005	pure	?
Source	Fibrous plants	Fibrous plants	Plant fibre	pure cellulose	Soley Wood
Other	Purified and depolymerized	Purified and depolymerized	Purified	Anionic polymer	Extracted 1.8% Lignin
Supplier	F.M.C. Corp. Philad., USA.	F.M.C. Corp. Philad., USA.	Brown Corp. N.Y., N.Y., USA.	Hericules Incorp., Oak Brook, IL., USA.	Westvaco, N. Charls., S.C., USA.

Appendix 2 (contd.)

PROPERTIES	PECTIN		LIGNIN	STARCH	
	LM - 3446	NF - 3442		TEXREX EXTENDER G	CELCA-SEC 500
Fibre Length	60 mesh	60 mesh	-	-	-
Colour	Cream	Cream	Dark Brown	White	White
Texture	Powder	Powder	Powder	Coarse flakes	Powder
Solubility (H <sub>2</sub> O)	Soluble	Soluble	Insoluble	Soluble	Soluble
Ash (%)	-	-	1.5	0.5	-
Moisture (%)	6 - 12	10	6	10	10
Heavy Metals (%)	-	< 0.001	-	-	-
Source	Citrus Fruit	Citrus Fruit	Pine Wood	Potato	Potato
Other	Low Ester	High Ester	Indulin 'AT' (Kraft)	Fractionation Product	Amylose Derivative
Supplier	Sunkist, Ontario, Cal., USA.	Sunkist Ontario, Cal., USA.	Westvaco, N. Charles., S.C., USA.	Celanes Co., Charlotte, N.C., USA.	Celanes Co., Charlotte, N.C., USA.

Appendix 3

Physico-Chemical Properties  
of Wheat Bran and Carrot

Properties	Wheat Bran (AACC)	Carrot (FAK)
Crude Fibre	8.9%	-
Water Insoluble Fibre	-	3.5%
Neutral Detergent Fibre	40.2%	1.5%
Acid Detergent Fibre	11.9%	1.4%
Lignin	3.2%	0.1%
Pectin	3.0%	2.0%
Protein	14.3%	1.1%*
Fat	0.2%	0.2%*
Carbohydrate (starch)	17.4%	8.1%*
Moisture	10.4%	88.6%*
Ash	5.1%	0.3%*
Sodium (mg/g)	1.0	50.0 *
Potassium (mg/g)	13.8	311.0 *
Calcium (mg/g)	1.2	37.0 *
Magnesium (mg/g)	4.3	21.0 *
Water Holding Capacity (g/g)	9.5	27.2
Cation Exchange (mmol Na/g)	-	1.6

\* Values taken from Documenta Giegy (1973)

#### Appendix 4

##### Description of Chemicals Used

<u>Name of Chemical</u>	<u>Source</u>	<u>Purity (Grade)</u>
Acetic Acid (glacial)	B.D.H. Chem. Ltd., Poole, Eng.	Analytical
Acetic Anhydride	B.D.H. Chem. Ltd., Poole, Eng.	Analytical
Acetone	Koch-Light Lab. Ltd., Colnbrook, Bucks, Eng.	Analytical
Barium Hydroxide	B.D.H. Chem. Ltd., Poole, Eng.	Analytical
Benzene	B.D.H. Chem. Ltd., Poole, Eng.	Analytical
Benzoic Acid	B.D.H. Chem. Ltd., Poole, Eng.	Analytical
p -Bromoaniline	B.D.H. Chem. Ltd., Poole, Eng.	Laboratory
Black Currant Syrup	Boots Co. Ltd., Nottingham, U.K.	B.P.C.
Chenodeoxycholic Acid	Maybridge Chem. Co. Ltd., Trevillet, Tintagel, Cornwall, U.K.	Analytical
Cholic Acid	Maybridge Chem. Co. Ltd., Trevillet, Tintagel, Cornwall, U.K.	Analytical
Chromium Trioxide	B.D.H. Chem. Ltd., Poole, Eng.	Analytical
Deoxycholic Acid	Maybridge Chem. Co. Ltd., Trevillet, Tintagel, Cornwall, U.K.	Analytical



Appendix 4 (contd.)

<u>Name of Chemical</u>	<u>Source</u>	<u>Purity (Grade)</u>
Diethyl Ether	Macfarlane Smith Ltd., Wheatfield Road, Fd.	Technical
3 $\alpha$ , 6 $\beta$ -Dihydroxy- 5 $\beta$ -Cholanoic Acid	Steraloids Inc., Wilton, N.H., U.S.A.	Analytical
2,2-Dimethoxypropane	Aldrich Chem. Co., Inc., Milwaukee, Wis., U.S.A.	Technical
Glucose	Evans Medical Ltd., Speke, Liv., Eng.	Biochemical
Hydrochloric Acid	B.D.H. Chem. Ltd., Poole, Eng.	Analytical
Hydrogen (gas)	British Oxygen Co., London.	-
Hyocholic Acid	Steraloids Inc., Wilton, N.H., U.S.A.	Laboratory
Hyodeoxycholic Acid	Steraloids Inc., Wilton, N.H., U.S.A.	Laboratory
Isoctane	B.D.H. Chem. Ltd., Poole, Eng.	Analytical
Isopropanol	B.D.H. Chem. Ltd., Poole, Eng.	Analytical
Lactose	Evans Medical Ltd., Speke, Liv., Eng.	Biochemical
Methanol	Koch-Light Lab. Ltd., Colnbrook, Bucks, Eng.	Analytical
Molecular Sieve ('5A')	Phase Separation Ltd., Flintshire, U.K.	-
$\alpha$ -Muricholic Acid	Rege Institute for Chemical Res., Leuven, Belgium.	-

# Appendix 4 (contd.)

<u>Name of Chemical</u>	<u>Source</u>	<u>Purity (Grade)</u>
<i>p</i> -Muricholic Acid	Rege Institute for Chemical Res., Leuven, Belgium.	-
<i>M</i> -Muricholic Acid	Prepared from rat colon content at our laboratory	> 95%
Nitrogen (gas)	British Oxygen Co., Ed.	Oxygen free
23-Nordeoxycholic Acid	Maybridge Chem. Co. Ltd., Trevillet, Tintagel, Cornwall, U.K.	Analytical
OV-17 (3%) - 100/120 Gas-Chrom Q (Phenylmethylsilicone)	Phase Separations, Flintshire, G.B.	High performance
Petroleum Spirit (60-80%)	Koch-Light Lab. Lt., Colnbrook, Bucks, Eng.	Analytical
Polyethylene Glycol (6000)	B.D.H. Chem. Ltd., Poole, Eng.	-
Perchloric Acid	B.D.H. Chem. Ltd., Poole, Eng.	Analytical
Pyrene	B.D.H. Chem. Ltd., Poole, Eng.	Analytical
3% SE-30-100/120 Supelcoport (Methyl silicone)	Supelco Inc., Bellafonte, Pen., U.S.A.	High performance
Silica Gel	Supelco Inc., Bellafonte, Pen., U.S.A.	-
Soda Lime	Medical Industrial & Equipment Ltd., Manchester, Eng.	-

# Appendix 4 (contd.)

<u>Name of Chemical</u>	<u>Source</u>	<u>Purity (Grade)</u>
Sodium Chloride	B.D.H. Chem. Ltd., Poole, Eng.	Analytical
Sodium Sulphate (anhydrous)	B.D.H. Chem. Ltd., Poole, Eng.	Analytical
Standard Gases ( $H_2$ & $CH_4$ )	Rank Precision Ind. Ltd., Kent, U.K.	In $N_2$
Sulphuric Acid	B.D.H. Chem. Ltd., Poole, Eng.	Analytical
Sylon HTP	Supelco Inc., Bellafonte, Pen., U.S.A.	-
Thiourea	B.D.H. Chem. Ltd., Poole, Eng.	Analytical
Toluene	B.D.H. Chem. Ltd., Poole, Eng.	Analytical
Ursodeoxycholic Acid	Steraloids Inc., Wilton, N.H., U.S.A.	Laboratory
D-Xylose	B.D.H. Chem. Ltd., Poole, Eng.	Biochemical
Zinc Sulphate	B.D.H. Chem. Ltd., Poole, Eng.	Analytical

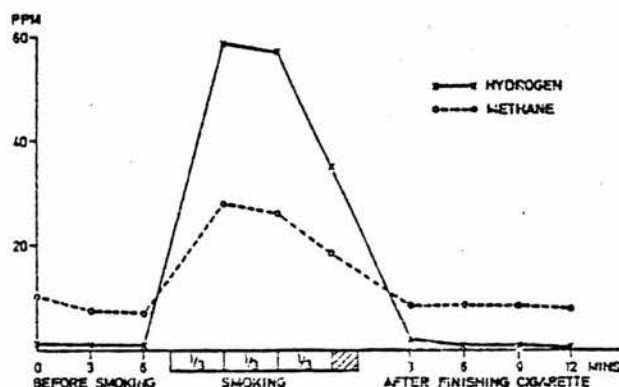
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## BREATH-HYDROGEN TEST AND SMOKING

SIR,—The breath-hydrogen test is a simple, non-invasive clinical test for diagnosis of disaccharidase deficiency,<sup>1</sup> intestinal bacterial colonisation,<sup>2</sup> and other diseases. It is expected that the method will become widely used as a diagnostic tool in the coming years. One suggestion is to take only one sample of breath for mass surveys.<sup>3</sup> We would like to share our experience with a pilot study showing a possible source of false-positive values.

We were alerted to the problem when doing breath hydrogen ( $H_2$ ) and methane ( $CH_4$ ) tests on a patient. The results showed high values of  $H_2$  and  $CH_4$ , which could have been easily interpreted as positive if a single sample had been taken, but the multiple samples showed irregularity in the pattern of excretion. This was later related to the patient's smoking in between sample taking. Not being aware that smoking is a source of error, we investigated the excretion pattern of  $H_2$  and



Mean values (five smokers) for breath  $H_2$  and  $CH_4$  before, during, and after smoking a cigarette.

$CH_4$  in a single end-expiratory sample of breath<sup>1</sup> from five smokers who inhaled. We took samples intermittently before, during and after smoking.

As the figure shows, sampling during a session of smoking will introduce an error in the  $H_2$  and  $CH_4$  values which wears off quickly, presumably as the smoke is washed out of the lung. Being a cigarette smoker per se does not seem to alter  $H_2$  and  $CH_4$  excretion, but breath samples taken within moments of smoking could give a false-positive value as a result of contamination with the smoke. That the source of error is the smoke and not the effect of smoking on  $H_2$  and  $CH_4$  production and/or excretion is supported by the fact that the pattern of  $H_2$  and  $CH_4$  excretion in the breath of smokers (when not smoking) is the same as that of non-smokers and the contaminatory effect of the smoke disappears rapidly, and by direct analysis of cigarette smoke.

We do not know whether these gases detected are actually  $H_2$  and  $CH_4$  or other volatile substances in cigarette smoke having the same retention-time in the gas-chromatographic analysis. However, it is clear that cigarette smoke can be a source of error in breath  $H_2$  and  $CH_4$  determination. We suggest that smoking should be avoided for at least 10 min before breath-sample taking.

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## Metabolism of dietary fibre components in man assessed by breath hydrogen and methane

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1. Breath hydrogen and methane were measured in eight normal individuals after acute and separate administration of different chemical components of dietary fibre.

2. Hemicellulose, raffinose and lactulose increased  $H_2$  production, while cellulose, pectin and lignin did not. Methane production was found to be individual and unaffected by any of the substances. Differences in physical properties of the same chemicals appear to have no influences on  $H_2$  and  $CH_4$  production.

Dietary fibre can be defined as that component of plant cell resistant to human alimentary enzyme action (Trowell, 1974). However, some studies on fibre show some hydrolysis by colonic bacteria, but, which components of fibre are hydrolysed and the extent of their breakdown in man is not clear (Williams & Olmsted, 1936). During anaerobic bacterial metabolism gaseous hydrogen ( $H_2$ ) and methane ( $CH_4$ ) are produced. These gases either pass in flatus or are excreted in the expired breath. Since mammalian cells do not produce  $H_2$  and  $CH_4$  the measurement of these gases in the breath could be used as a simple and non-invasive measure of the intestinal fermentation of dietary fibre (Newman, 1974).

In this study we were interested to see the degree of breakdown of individual chemical components of dietary fibre when given separately to fasted individuals and the response followed over a short period.

### MATERIALS AND METHODS

Participants in the study were eight normal volunteers (four males and four females), 23 to 47 years of age. They had no gastrointestinal problems and were in good health.

The procedure involved fasting for 15 h from 24.00 hours to 15.00 hours the following day. Ten or 20 g of the fibre component were administered raw in 400 ml water with blackcurrant syrup as flavouring (B.P.C., Boots Co. Ltd, Nottingham). The meals were taken at about 09.00 hours. The time interval between each experiment was at least 1 week. Substances studied were the oligosaccharide raffinose (crystalline: BDH Chemicals, Poole, Dorset), the polysaccharides fibrous cellulose (Solka-Floc SW-40, Brown Corporation, New York), microcrystalline  $\alpha$ -cellulose (FMC Corporation, Philadelphia), sodium carboxymethyl cellulose (type 7HF, Hercules Corporation, Illinois), hemicellulose (from Soley Hardwood – RLX 4121-36, Charleston Research Center, Westvaco), pectin NF 3442 high methyl and pectin LM 3466 low methyl content (Citrus Growers Incorporation, California), lignin (Indulin AT: Kraft processed pinewood, Westvaco, Charleston, South Carolina). The control pattern was established with 400 ml water containing blackcurrant juice flavouring. Additional controls were the non-absorbable artificial disaccharide lactulose 50% solution (Duphar Laboratories, Southampton) and the starches: Textrex extender G and Celca-Sec 500, both potato based (Celanese, Charlotte, North Carolina).

End-expiratory breath samples were taken by a modified Haldane-Priestly Alveolar

Table 1. *Breath hydrogen ( $\mu\text{mol/l}$ ) from subjects given different chemical components of dietary fibre*

(Mean values and standard deviations)

Chemical 20 g	No. of subjects	Peak		Total (6 samples)		
		Mean	SD	Mean	SD	
Control	8	0.27	0.14	1.00	0.73	
Starch	7	0.27	0.32	0.86	1.14	NS
Cellulose	7	0.36	0.23	1.09	1.09	NS
Hemicellulose*	5	0.77	0.59	1.82	1.32	$P < 0.05$
Pectin	6	0.36	0.23	1.18	1.09	NS
Lignin	5	0.36	0.18	0.91	0.59	NS
Raffinose*	6	2.05	0.77	5.64	2.09	$P < 0.01$
Lactulose	6	2.18	1.46	7.64	4.68	$P < 0.005$

NS, not significant.

\* Ten g only in sample.

Table 2. *Total breath methane ( $\mu\text{mol/l}$ ) from subjects given different chemical components (20 g) of dietary fibre (6 samples)*

Subject	Control	Starch	Cellulose	Hemi-cellulose*	Pectin	Lignin	Raffinose*	Lactulose
G.P.	11.50	10.96	13.86	—	—	12.73	10.55	11.50
A.S.	1.14	0.18	0.09	2.18	0.41	—	0.73	0
D.S.	0	0.09	0.09	0.50	—	0	0.23	0

\* Ten g only administered

sampling method (Metz, Gassul, Leeds, Blendis & Jenkins, 1976), at hourly intervals over the test period.  $\text{H}_2$  and  $\text{CH}_4$  in the breath samples were measured by gas chromatography (Pye Series 104) using molecular sieve packed glass columns and a katharometer. Carrier gas was nitrogen (oxygen free) at flow rate of 65 ml/min; oven temperature,  $50^\circ$ ; detector temperature,  $100^\circ$ ; bridge current, 140 mA; sensitivity for 1  $\mu\text{mol/l}$  of  $\text{H}_2$  and 1  $\mu\text{mol/l}$   $\text{CH}_4$ , 10% of full scale deflection of recorder; analytical reproducibility, coefficient of variation less than 3%,  $n$  20.

Measurements used for comparison were the peak  $\text{H}_2$  and  $\text{CH}_4$  (maximal value recorded) and the total  $\text{H}_2$  and  $\text{CH}_4$  (summation of all values recorded), and expressed in  $\mu\text{mol/l}$ .

## RESULTS

*Subjective symptoms.* Some subjects experienced abdominal distension and variable amounts of gaseousness, particularly with lactulose, raffinose and hemicellulose in that order. One participant had diarrhoea with lactulose.

*Transit time.* The average time for the breath  $\text{H}_2$  to increase after lactulose was 1.5–2 h.

*Breath  $\text{H}_2$ .* As shown in Table 1 the base line control values are peak  $0.27 \pm 0.14$   $\mu\text{mol}$  ( $0.14$ – $0.46$   $\mu\text{mol/l}$ ) and total  $1.00 \pm 0.73$   $\mu\text{mol}$  ( $0.23$ – $1.91$   $\mu\text{mol/l}$ ). Starch, cellulose, pectin and lignin did not significantly alter the excretion pattern. Hemicellulose (10 g) produced a peak excretion of  $0.77 \pm 0.59$   $\mu\text{mol}$  ( $0.14$ – $1.36$   $\mu\text{mol/l}$ ) and a total  $1.82 \pm 1.32$   $\mu\text{mol/l}$  ( $0.18$ – $3.59$   $\mu\text{mol/l}$ ). Raffinose (10 g) and lactulose (20 g) considerably increased gas production, with the breath  $\text{H}_2$  reaching values of 2.27  $\mu\text{mol/l}$  and over for the peak and 3.18–16.36  $\mu\text{mol/l}$  total.

*Breath  $\text{CH}_4$ .* Base line measurements showed that of the eight participants, three excreted more than 0.91  $\mu\text{mol/l}$ , two excreted about 0.46  $\mu\text{mol/l}$  and three did not excrete any. Table 2 summarizes the total  $\text{CH}_4$  excretion following the administration of the different



substances for a representative of each of the three groups. There was no appreciable change in excretion for any of the substances.

*Differences of physical and chemical Properties.* The ingestion of polysaccharides with different physical properties (i.e. particle size) and some chemical changes of the following substances, [cellulose [AV-101 (50); AV-102 (100); SW-40 (140); Na.COOH-Me]; pectin [LM-3466 (low ester); NF-3442 (high ester)]; starch [C-S 500 (fine); T-EG (coarse)], did not make significant changes in the expired  $H_2$  and  $CH_4$ .

#### DISCUSSION

Anaerobic bacterial breakdown of dietary fibre components produce the gases  $H_2$  and  $CH_4$  as some of the final products of their metabolism (Gray & Gest, 1965; Calloway, Colasito & Mathew, 1966). Our acute study indicates that few of the different components of dietary fibre when ingested on a single occasion result in the production of  $H_2$  and  $CH_4$ . This might imply that the substances are not being metabolized. Alternatively they could have been metabolized along other pathways, for example partially degraded to volatile fatty acids and other short chain products (Williams & Olmsted, 1936; Cummings, 1973).

Starch did not produce  $H_2$  and  $CH_4$ , presumably because of hydrolysis by  $\alpha$ -amylase in the upper part of the small intestine. Lignin is resistant to hydrolysis by colonic bacteria (Cummings, 1973; Southgate, Branch, Hill, Drasar, Walters, Davies & Baird, 1976). It was expected that pectin, which is reputed to be extensively hydrolysed, would result in an increased  $H_2$  and  $CH_4$  production (Werch & Ivy, 1941; Cummings, 1976). This did not happen and this may be due to hydrolysis along paths not leading to  $H_2$  production. Modest production of  $H_2$  by hemicellulose may be due to the comparatively small amount used (10 g) yet the gas production is not comparable to 10 g raffinose.

The mouth to caecum transit time may be a variable both between individuals and chemicals. Neither of these seem to apply as the lactulose transit time was comparable in all subjects (1.5–2 h) and prolonged following of the breath gases for 10 to 12 h rendered no delayed increase.

The  $CH_4$  excretion pattern remained individual as has been previously observed (Calloway & Murphy, 1969; Bond, Engel & Levitt, 1970). But what is difficult to explain is the lack of increased production by  $CH_4$  excretors, who presumably have the appropriate bacteria, when provided with the correct substrate.

The physical nature of the substances may be a factor affecting bacterial hydrolysis. We looked into this in our study particularly with cellulose and pectin. Differences in physical property (i.e. particle size) and some chemical change appear to make no appreciable change in the  $H_2$  excretion pattern. This also applies to  $CH_4$  excretion.

We would like to thank the World Health Organisation for assistance with a fellowship (K.T.) and Vitamins Inc., Chicago for financial assistance and provision of fibre isolates.

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## Note

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### Gas chromatographic technique for combined measurement of hydrogen and methane using thermal conductivity detector

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(First received September 11th, 1978; revised manuscript received October 30th, 1978)

Breath hydrogen and methane measurement is a useful diagnostic and research tool for studying bacterial activity in the colon<sup>1</sup>. Since it is believed that a katharometer detector (KD) is not sufficiently sensitive for methane measurement, the methods used for the gas chromatographic analysis of these gases employ different detectors for each gas, *i.e.* katharometer for hydrogen and flame-ionization detector (FID) for methane<sup>2</sup>. As a result of this and presumably due to lack of simultaneous access to both systems, most workers measure only one of the two gases. As a diagnostic tool, the value of measuring methane compared with hydrogen is at present minimal. However, recently one possible and important diagnostic use of methane has been suggested<sup>3</sup> and it is possible that others will emerge. A combined measurement using a single detection system would facilitate application and further investigation of these related gases. In this paper a method of detection of both gases in one breath sample using only a KD is described.

### MATERIALS AND METHODS

Samples of alveolar air are collected in syringes via a modified Haldane-Priestly tube<sup>4</sup>. The sample of air is analysed using a gas-solid chromatograph (Pye Series 104) with molecular sieve packed glass columns and a KD. The sample is injected by means of a sampling loop, after passage through a tube containing soda lime and silica gel (in series) to remove carbon dioxide and water, respectively. Values are displayed on a 1-mV pen recorder (Philips, PM-8000). Two known concentrations 0.9  $\mu\text{mole/l}$  and 4.5  $\mu\text{mole/l}$  of hydrogen and methane mixtures in nitrogen are used as standard gases (Rank Hilger, Kent, Great Britain). The standard gases are analysed each day during the processing of test samples, to check the sensitivity and stability of the instrument.

The effect of changing each chromatographic variable (*e.g.* detector temperature, bridge current, carrier gas) on sensitivity and peak resolution were determined by keeping other variables constant. Working conditions were chosen which both optimized the resolution and sensitivity for hydrogen and methane measurement and still remained within the limits of the manufacturer's recommendations.

The sensitivity of the system was assessed using standard gases at different concentrations and the recorded deflection plotted against concentration.

The analytical precision was evaluated in two ways. One by analysing 20 samples of each standard gas consecutively, the other by repeated analysis of 30 different breath samples with a wide range of hydrogen and methane concentrations.

## RESULTS

Changing the carrier gas from argon to nitrogen (oxygen free) resulted in good resolution of the methane peak (peak resolution between oxygen and methane = 6). Increasing the detector temperature and bridge current, improved detector sensitivity to both gases particularly methane. A finer stationary phase improved the separation between hydrogen and oxygen peaks.

Optimum working conditions and chromatograms of 4.5  $\mu\text{mole/l}$  standard gas and a sample of breath from a person with high hydrogen and methane excretion (1.2 and 1.6  $\mu\text{mole/l}$ , respectively) are shown in Fig. 1. The retention time for hydrogen was about 65 sec and methane about 300 sec. Peak resolution between hydrogen and oxygen was 3.3 and that between oxygen and methane 6.0.

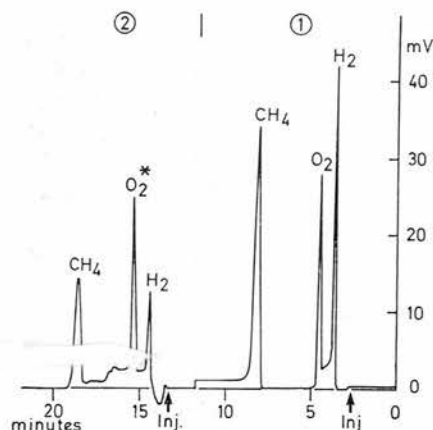


Fig. 1. Chromatograms (1), 4.5  $\mu\text{mole/l}$  standard gas and (2), breath sample. Conditions: column, 2.6 m  $\times$  4.0 mm I.D. glass, packed with 60–85 mesh molecular sieve 5A; temperature, 50°; carrier gas, oxygen-free nitrogen at 65 ml/min; detector, katharometer, 140 mA bridge current, temperature 100°; chart speed, 5 mm/min; attenuation, set at 2 ( $\times$  500). \* set at 50 ( $\times$  20).

A linear relationship with a slope of 0.49 for hydrogen and 0.36 for methane was observed when different concentrations of the gases were plotted against recorder deflection. Repeat analysis ( $n = 20$ ) of 0.9 and 4.5  $\mu\text{mole/l}$  hydrogen and methane standards showed a coefficient of variation of 4% for both gases, while different concentrations (range hydrogen, 0–6.8  $\mu\text{mole/l}$ ; methane, 0–4.5  $\mu\text{mole/l}$ ) of breath samples on different analytical batches ( $n = 30$ ) showed a coefficient of variation of 1% for hydrogen and 7% for methane.

## DISCUSSION

The essential difference in the method used here is the use of nitrogen (oxygen free) as a carrier gas. Most workers use either argon or helium. These gases are ideal for hydrogen measurement and are recommended for such purpose. However, if hydrogen and methane require to be simultaneously measured and if one of these gases is used as carrier, two detector systems (*i.e.* KD and FID) are needed. This is because of the large breath nitrogen peak which obliterates the methane peak. Using nitrogen as carrier gas the breath nitrogen peak is eliminated and good methane peak resolution is achieved. Also, nitrogen can be used at a higher bridge current than argon and this is an advantage for reasons described above.

As the chromatograms in Fig. 1 show, distinct and well resolved peaks are displayed. The elution time of about 6 min is comparable to the time taken to process a single breath sample for hydrogen alone, using the usual carrier gases<sup>2</sup>. The response of the instrument is linear for the range of concentrations usually encountered in practice (*i.e.* 0 to 4.5  $\mu\text{mol/l}$ ). The modified attachment to remove carbon dioxide and water from the samples helps to minimise the requirement for purging the stationary phase and gives less noisy peaks.

## ACKNOWLEDGEMENTS

We would like to thank the World Health Organization for assistance with a Fellowship (K.T.) and Mr. Louis Kovacs and Vitamins Inc., Chicago, for financial assistance.

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## Note

### Gas chromatographic procedure for the measurement of bile acids in rat intestine

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(First received September 5th, 1978; revised manuscript received November 16th, 1978)

In rat liver, chenodeoxycholic acid is metabolized to  $\alpha$ - and  $\beta$ -muricholic acids, which are secreted in the bile\*. In the colon some bile acids are metabolized to hyodeoxycholic acid and  $\omega$ -muricholic acid<sup>1</sup>, both of which are excreted in considerable amounts in rat faeces. In order to study the metabolism of bile acids in the rat it is necessary to identify and estimate these bile acids separately.

The separation of bile acid methyl ester acetates of lithocholic, deoxycholic, chenodeoxycholic, cholic and hyodeoxycholic acid has been described<sup>2</sup>. The bile acids are eluted in that sequence when 2.25% SE-30 silicone rubber gum on Chromosorb W (60-80 mesh) is used, the less polar hyodeoxycholic acid being retained more strongly than cholic acid. Neither OV-17 nor OV-225 separates the muricholic acid methyl ester acetates satisfactorily. When OV-17 is used  $\omega$ - and  $\beta$ -muricholic acid methyl ester acetates are not adequately separated (peak resolution: 0.4); and when OV-225 is used  $\alpha$ - and  $\omega$ -muricholic acid methyl ester acetates are not adequately separated (peak resolution: 0.5).

Bile acid trimethylsilyl (TMS) ethers have been separated on Hi-eff-8B<sup>3</sup>, but  $\alpha$ -muricholic and cholic acids are not resolved in this system. The separation of the TMS ethers of cholic and  $\alpha$ -,  $\beta$ - and  $\omega$ -muricholic acids on OV-17 is described<sup>4</sup>, but chenodeoxycholic and deoxycholic acids are not resolved in this system. It has been reported<sup>5</sup> that TMS ether derivatives are not stable and that this can lead to multiple peaks from individual trihydroxy bile acids. In this paper a procedure is described which separates the following bile acids in sequence: lithocholic, deoxycholic, chenodeoxycholic, cholic, hyodeoxycholic, hyocholic,  $\alpha$ -muricholic,  $\omega$ -muricholic, and  $\beta$ -muricholic acids.

\* Common and systematic names of compounds mentioned in the text are as follows: nordeoxycholic acid, 23-nor-5 $\beta$ -cholanoic acid-3 $\alpha$ ,12 $\alpha$ -diol; lithocholic acid, 5 $\beta$ -cholanoic acid-3 $\alpha$ -ol; deoxycholic acid, 5 $\beta$ -cholanoic acid-3 $\alpha$ ,12 $\alpha$ -diol; chenodeoxycholic acid, 5 $\beta$ -cholanoic acid-3 $\alpha$ ,7 $\alpha$ -diol; ursodeoxycholic acid, 5 $\beta$ -cholanoic acid-3 $\alpha$ ,7 $\beta$ -diol; hyodeoxycholic acid, 5 $\beta$ -cholanoic acid-3 $\alpha$ ,6 $\alpha$ -diol; cholic acid, 5 $\beta$ -cholanoic acid-3 $\alpha$ ,7 $\alpha$ ,12 $\alpha$ -triol; hyocholic acid, 5 $\beta$ -cholanoic acid-3 $\alpha$ ,6 $\alpha$ ,7 $\alpha$ -triol;  $\alpha$ -muricholic acid, 5 $\beta$ -cholanoic acid-3 $\alpha$ ,6 $\beta$ ,7 $\alpha$ -triol;  $\beta$ -muricholic acid, 5 $\beta$ -cholanoic acid-3 $\alpha$ ,6 $\beta$ ,7 $\beta$ -triol;  $\omega$ -muricholic acid, 5 $\beta$ -cholanoic acid-3 $\alpha$ ,6 $\alpha$ ,7 $\beta$ -triol.

## MATERIALS AND METHODS

Cholic, chenodeoxycholic, deoxycholic, lithocholic and 23-nordeoxycholic acids were obtained from Maybridge Chemical Co., Tintagel, Great Britain. Hyodeoxycholic, hyocholic, ursodeoxycholic and 3 $\alpha$ ,6 $\beta$ -dihydroxy-5 $\beta$ -cholanoic acids were obtained from Steraloids, Wilton, N.H., U.S.A. The  $\alpha$ - and  $\beta$ -muricholic acid methyl esters were a generous gift from Professor H. Eyssen, Rega Institute for Medical Research, Leuven, Belgium.  $\omega$ -Muricholic acid methyl ester was prepared from rat colon by thin-layer chromatography (TLC)<sup>6</sup> and identified by gas-liquid chromatography (GLC) as a TMS ether on OV-17<sup>4</sup> and SE-30<sup>6</sup>.

The small-intestinal and colonic contents of rat were washed out with distilled water, freeze-dried, and weighed. Bile acids and salts were extracted, hydrolysed, and partially purified according to Evrard and Janssen<sup>7</sup>. Following extraction with diethyl ether, bile acids were methylated using a dimethoxypropane-methanol-conc. HCl mixture (50:20:1). It was necessary to further purify methyl esters of colonic bile acids by TLC. Methyl ester acetates were then formed by a modification of the method of Roovers *et al.*<sup>8</sup>. The methyl esters were acetylated for 2 h at 0° with 1 ml of a mixture of acetic acid-acetic anhydride-70% HClO<sub>4</sub>. (700:500:1). At the end of this period, 10 ml of a 20% (w/v) solution of NaCl in water was added, and the methyl ester acetates were extracted with three portions of diethyl ether. The pooled extracts were washed with distilled water and then taken to dryness.

Separation of the methyl ester acetate derivatives was carried out on a Pye Unicam Series 104 dual-column chromatograph, equipped with hydrogen flame ionisation detectors. Five-ft. columns were silanized, and packed with 3% SE-30 on 100-120 mesh Supelcoport (Supelco, Bellefonte, Pa., U.S.A.). Nitrogen (oxygen free) was used as carrier gas at a flow-rate of 35 ml/min, and inlet pressure 206 kPa (30 p.s.i.). The operating temperature for column and detector was 262°, and the inlet temperature was 280°. The internal standard was 23-nordeoxycholic acid.

TABLE I

RELATIVE RETENTION TIMES OF BILE ACID METHYL ESTER ACETATES ON 3% SE-30 AT 262°

Peak no.	5 $\beta$ -Cholanoic acid	Retention time relative to 23-nordeoxycholic acid
1	23-nor-3 $\alpha$ ,12 $\alpha$ -diol	1.00
2	3 $\alpha$ -ol	1.06
3	3 $\alpha$ ,12 $\alpha$ -diol	1.34
4	3 $\alpha$ ,7 $\alpha$ -diol	1.55
5	3 $\alpha$ ,7 $\alpha$ ,12 $\alpha$ -triol	1.69
6	3 $\alpha$ ,6 $\alpha$ -diol	1.89
7	3 $\alpha$ ,6 $\beta$ -diol	1.97
7	3 $\alpha$ ,7 $\beta$ -diol	1.97
—	3 $\alpha$ ,6 $\alpha$ ,7 $\alpha$ -triol	2.28
8	3 $\alpha$ ,6 $\beta$ ,7 $\alpha$ -triol	2.41
9	3 $\alpha$ ,6 $\alpha$ ,7 $\beta$ -triol	2.82
10	3 $\alpha$ ,6 $\beta$ ,7 $\beta$ -triol	3.11



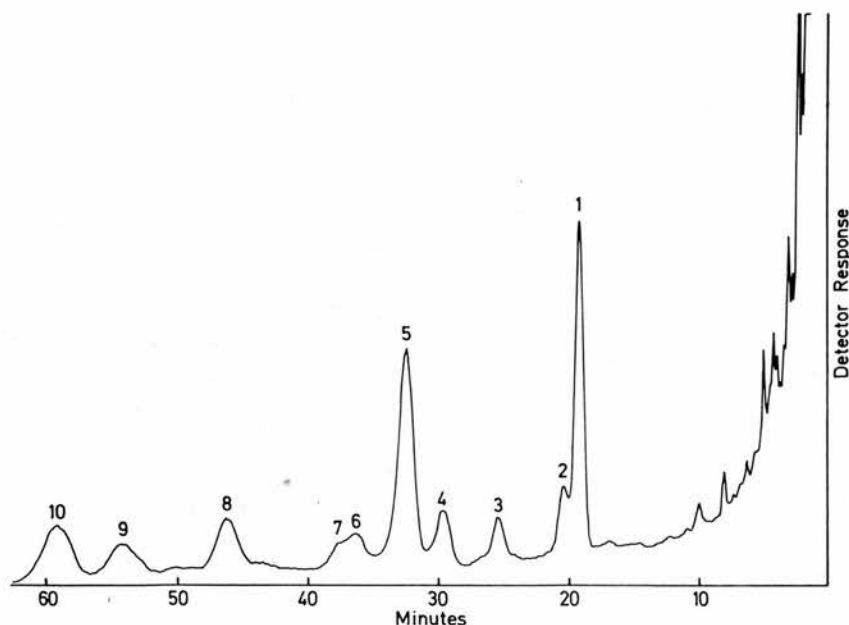


Fig. 1. GLC separation of the bile acid methyl ester acetates in rat intestine. Column, 3% SE-30 on 100–120 mesh Supelcoport; temperature, 262°. For peak identification see Table I.

## RESULTS

The retention times of the methyl ester acetate derivatives of bile acids relative to 23-nordeoxycholic acid are shown in Table I.

A gas chromatographic recording of total intestinal bile acid methyl ester acetates is shown in Fig. 1.

## ACKNOWLEDGEMENTS

We thank the World Health Organisation for assistance with a Fellowship (K.T.), and the Incorporated National Association of British and Irish Millers Ltd. for financial assistance.

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